PROTEIN INVOLVEMENT IN PROSTAGLANDIN PRODUCTION

BY THE GUINEA-PIG UTERUS

by

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In accordance with the requirements of regulation 3.4.7 this thesis has been composed by myself and the work presented herein is my own.

Caroline Leckie

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ABSTRACT OF THESIS (Regulation 3.5.10)

10mM sodium fluoride inhibited the output of prostaglandin (PG)$F_{2\alpha}$ from Day-7 guinea-pig endometrium and the outputs of $PGF_{2\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium during 24h of tissue culture. The incorporation of [^{3}H]-leucine into cellular and secreted proteins by Day-15 guinea-pig endometrium in culture was inhibited by over 90% by sodium fluoride (10mM). Thus, as far as endometrial PG and protein synthesis are concerned, the effects of sodium fluoride are similar to those of other protein synthesis inhibitors and provide further evidence that the increase in endometrial $PGF_{2\alpha}$ synthesis at the end of the oestrous cycle is dependent on the stimulation of fresh protein synthesis by oestradiol acting on a progesterone-primed uterus.

Over a shorter period of culture (1h), sodium fluoride (10mM) stimulated the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium. However, the other G-protein modulators, cholera and pertussis toxin, had no effect on the output of PGs from Day-7 or Day-15 guinea-pig endometrium in culture for up to 24h. Consequently, a toxin-insensitive G-protein may be involved in mediating PG release from guinea-pig endometrium. Sodium fluoride also increased the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 and Day-15 guinea-pig uterus superfused *in vitro*. The sodium fluoride-mediated increase in PG output from the superfused Day-7 uterus was unaffected by removal of extracellular calcium but was prevented by the intracellular calcium antagonist TMB-8. The calmodulin inhibitors, W-7 and trifluoperazine, and the phospholipase C inhibitor, neomycin, had no effect on the sodium fluoride-stimulated increases in PG output from the superfused Day-7 uterus. Therefore, sodium fluoride appears to stimulate uterine PG output by releasing calcium from intracellular stores, and this release is not due to activation of PLC nor is it mediated by calmodulin.

Purification of proteins secreted into culture medium by Day-15 guinea-pig endometrium by affinity, ion-exchange and gel filtration chromatography failed to isolate any protein fractions which affected PG output from Day-7 guinea-pig endometrium in culture. However, several low molecular weight (<20kDa) fractions were isolated which stimulated the activity of cobra venom PLA_2 *in vitro*. Cellular proteins isolated from Day-15 guinea-pig endometrium had no effect on the output of PGs from Day-7 endometrium in culture or on the activity of PLA_2 from cobra venom.

The output of PGs, particularly $PGF_{2\alpha}$ was found to be lower from pregnant than non-pregnant guinea-pig endometrium in culture on Day-15. Human α-interferon had no effect on the output of PGs from Day-15 guinea-pig endometrium in culture and there was no detectable antiviral activity, indicative of interferon-like proteins, secreted into culture medium by Day-15 guinea-pig conceptuses. Consequently, it is unlikely that the antiluteolytic mechanism in the pregnant guinea-pig is due to the secretion by the conceptus of interferon-like proteins as occurs in several species of domestic ruminant. Factors secreted into culture medium by Day-15 guinea-pig conceptuses and Day-15 pregnant guinea-pig endometrium and proteins purified from the culture medium of Day-15 guinea-pig conceptuses had no effect on the output of PGs from Day-15 non-pregnant guinea-pig endometrium in culture over 12h. Neither did proteins purified from the culture medium of Day-15 conceptuses affect the activity of cobra venom PLA_2. It is possible that the antiluteolytic factor from the guinea-pig conceptus is (i) produced earlier in pregnancy than Day-15, (ii) not secreted *in vitro*, (iii) present in very small amounts or (iv) acts other than by a direct effect on endometrial PG synthesis.

CONTENTS

SECTION 1

GENERAL INTRODUCTION

Prostaglandins historically

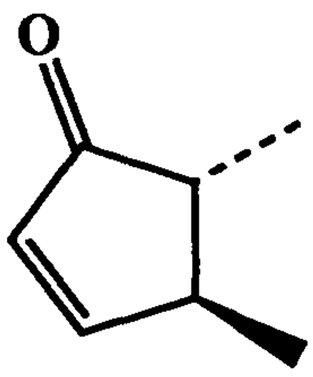
The link between prostaglandins and reproduction was first established 60 years ago when biological activity was identified in human semen and animal seminal tissues. Research being carried out into artificial insemination revealed that human seminal fluid could induce either relaxation or contraction of isolated human uterine strips (Kurzrok and Lieb, 1930). Previously, the strong depressor action of extracts of fresh human prostrate tissue on the blood pressure of dogs (Battez and Boulet, 1913) had been attributed to the known biologically and pharmacologically active compounds of that time such as acetylcholine, adenosine, bradykinin, kallikrein and substance P. However, after Goldblatt and Von Euler had independently demonstrated the presence of a vasodepressor agent and smooth muscle stimulating factor in human seminal plasma (Goldblatt, 1933; Goldblatt, 1935) and sheep vesicular glands (Von Euler, 1934), the biological activity of male reproductive tissue and fluid was found to be due to a new and previously unknown chemical which was surprisingly, as a physiological role for fatty acids had not previously been suspected, lipid soluble (Von Euler, 1936). The substance was named prostaglandin but, due to the inadequate purification methods and analytical techniques at that time and the very low amounts of prostaglandins present in tissues, the isolation of crystalline prostaglandin (PG)F and prostaglandin (PG)E from sheep prostate glands (Bergstrom and Sjovall, 1960; Bergstrom, Krabisch and Sjovall, 1960) was not achieved for another 25 years.

Prostaglandins chemically

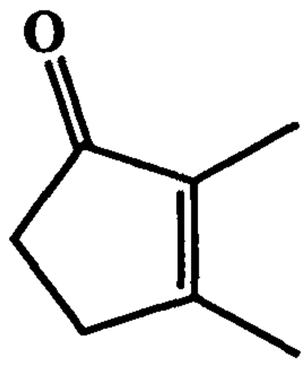
Once the prostaglandins were isolated, the chemical structures of PGE and PGF were quickly elucidated (Bergstrom, Ryhage, Samuelsson and Sjovall, 1963). The prostaglandins were found to be a family of structurally related compounds but with a wide diversity of biological effects. They are 20 carbon-containing, unsaturated hydroxy fatty acids consisting of a cyclopentane ring and 2 side chains of 7 and 8 carbons, respectively. They are divided into 3 "series" according to the number of double bonds in their side chains. Prostaglandins of the biologically important "2 series", or the dienoic prostaglandins, have double bonds between carbons 5 and 6 and between carbons 13 and 14. In addition dienoic prostaglandins have a hydroxyl group at carbon 15 and either a hydroxyl or a ketone group at carbon 9 and, with the exception of the A, B and C forms, at carbon 11. Prostaglandin (PG)I_2 is cyclised between carbon 6 and the oxygen moiety at position 9. Figure 1 illustrates the cyclopentane rings of prostaglandins A-F and thromboxane A, the structure of PGI_2 and a generalised carbon skeleton of a prostaglandin of the "2 series".

Prostaglandin biosynthesis

The precursors of prostaglandins are unsaturated fatty acids (Van Dorp, Beerthius, Nugteren and Vonkeman, 1964; Bergstrom, Danielsson and Samuelsson, 1964). In the case of the dienoic prostaglandins, this precursor is arachidonic acid which can be obtained from the diet or synthesised from the essential fatty acid linoleate by chain elongation and desaturation. Arachidonic acid is converted into the unstable, but biologically active cyclic endoperoxide intermediates, PGG_2 and PGH_2 (Hamberg, Svensson, Wakabayashi and Samuelsson,



PGA



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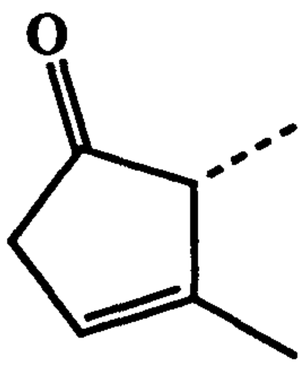

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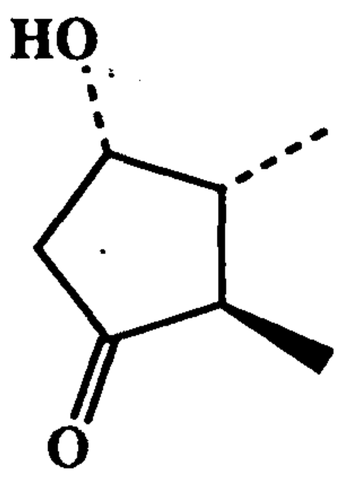
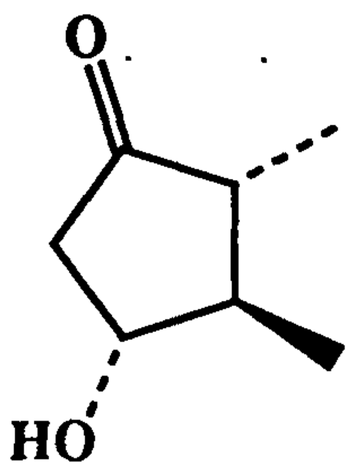
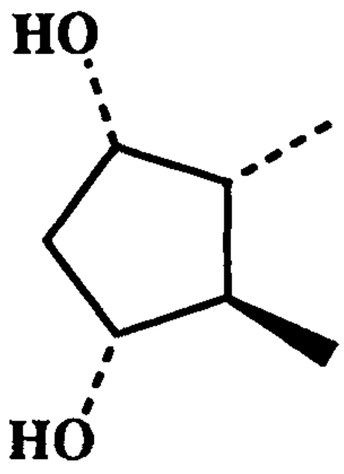


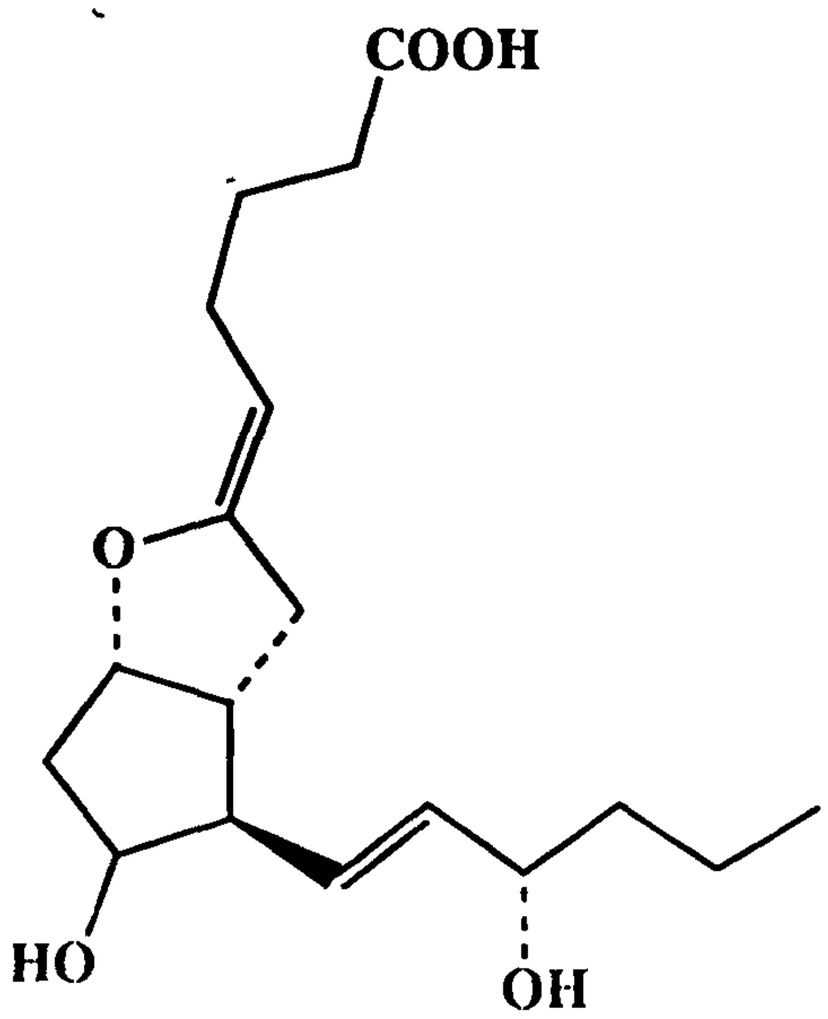
PGD



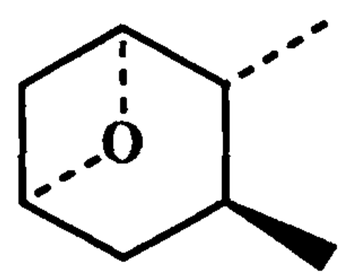
PGE



PGF_{α}



PGI_2



TXA

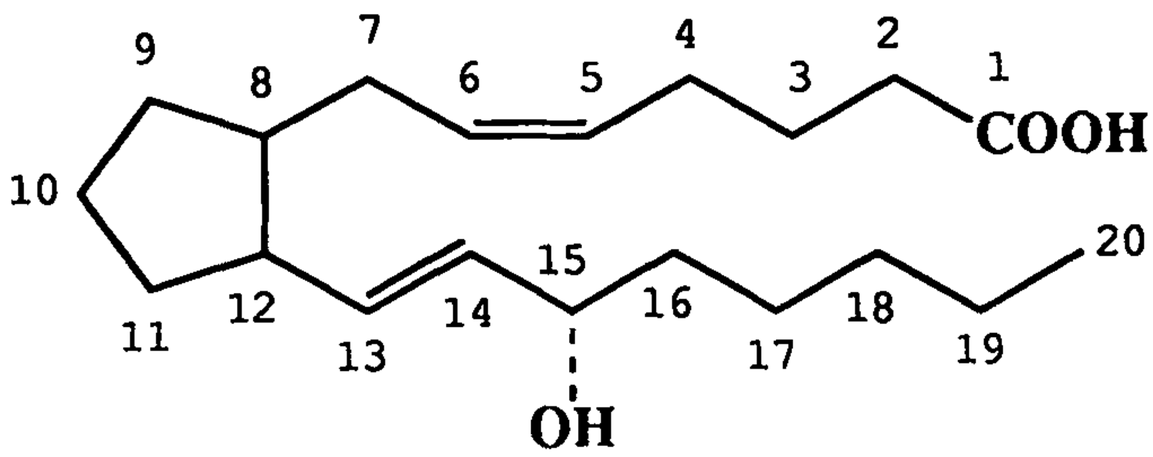


Fig. 1. The structures of the cyclopentane rings of prostaglandins A-F and thromboxane A, the structure of PGI_2 and a generalised carbon skeleton of a prostaglandin of the "2 series".

1974), by the action of the enzyme prostaglandin endoperoxide synthase which is part of the membrane bound "prostaglandin synthetase complex". The enzyme prostaglandin endoperoxide synthase has two activities, a fatty acid cyclo-oxygenase (which converts arachidonic acid to PGG_2) and prostaglandin hydroperoxidase (which converts PGG_2 to PGH_2). Other enzymes in the "prostaglandin synthetase complex" catalyse the isomerase and reductase reactions which lead to the formation of prostaglandins as well as the thromboxane compounds. Figure 2 illustrates the enzymes involved in the conversion of arachidonic acid to the prostaglandin endoperoxides PGG_2 and PGH_2 with the subsequent formation of the prostaglandin and thromboxane compounds.

Control of prostaglandin biosynthesis

The enzymes involved in the prostaglandin synthetase complex are found in almost every cell and the presence of prostaglandins has been demonstrated in many tissues (Bergstrom, 1966; Karim, Sandler and Williams, 1967). However, intracellular levels of free fatty acids are very low (Marcus, Ullman and Safier, 1969; Haye and Jacquemin, 1977) and fatty acids esterified to lipids are not substrates for oxidative cyclisation to prostaglandins (Lands and Samuelsson, 1968; Vonkeman and Van Dorp, 1968). Therefore, the hydrolysis of fatty acids to their non-esterified form must occur in order for prostaglandin biosynthesis to take place.

Accumulation of prostaglandins in tissues does not occur (Jouvenaz, Nugteren, Beerthius and Van Dorp, 1970), but prostaglandins form rapidly when tissues are removed and allowed to stand before extraction (Eliasson, 1959; Pace-Asciak and Wolfe, 1968; Vogt, Meyer, Kunze, Lufft and Babilli, 1969). This indicates

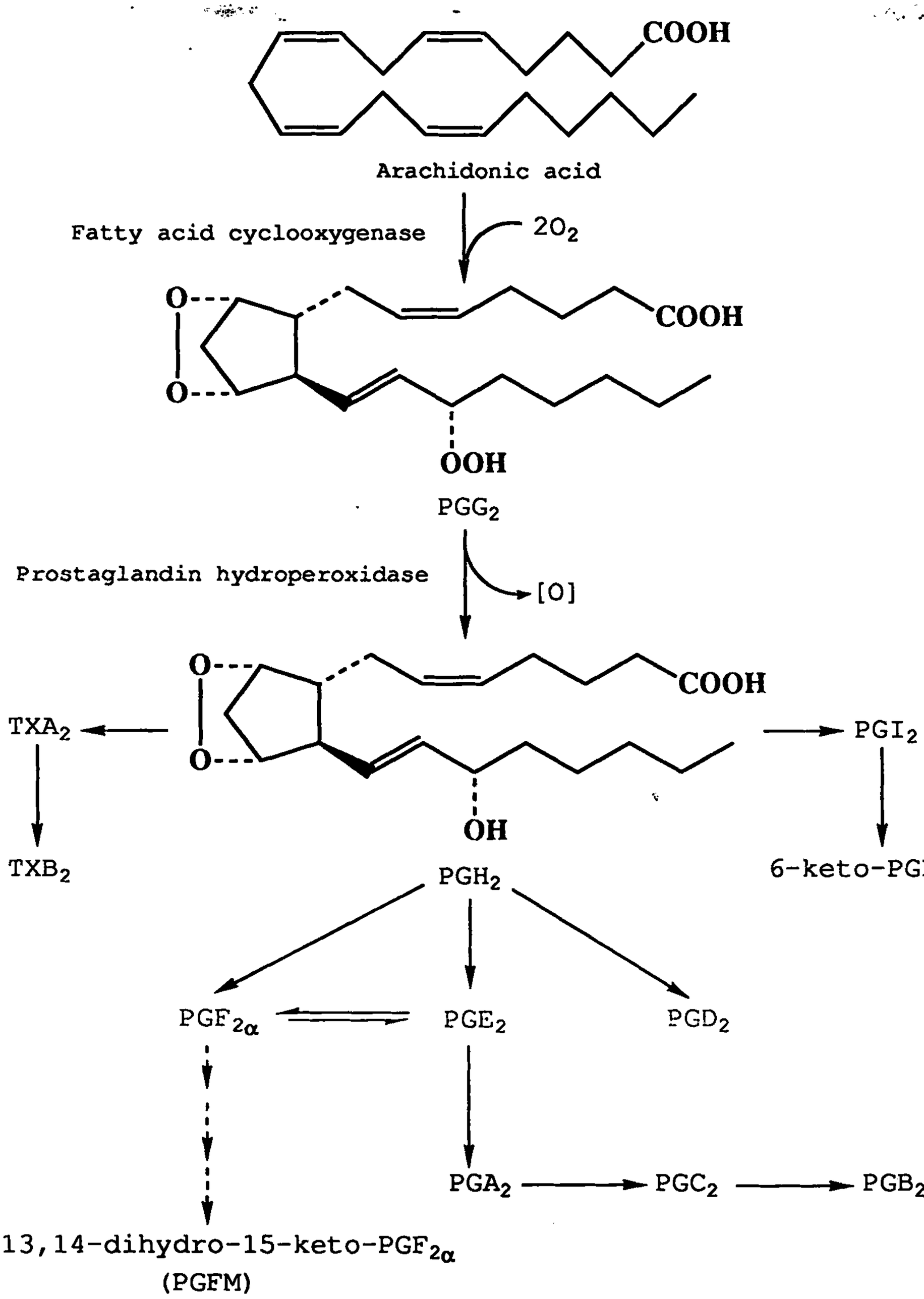


Fig. 2. The reactions and enzymes involved in the conversion of arachidonic acid to the prostaglandin endoperoxides PGG_2 and PGH_2 with the subsequent formation of the prostaglandin and thromboxane compounds. The enzymes fatty acid cyclooxygenase and prostaglandin hydroperoxidase together constitute prostaglandin endoperoxide synthase.

that prostaglandin release and action are intimately related to prostaglandin biosynthesis under physiological conditions. As the arrival of free fatty acid substrate at the prostaglandin synthetase complex is necessary for prostaglandin biosynthesis, the hydrolysis of fatty acids to their non-esterified form would appear to be an important control step in prostaglandin release.

Release of arachidonic acid from membrane lipids

There are a number of intracellular lipid pools which could be the source of precursor arachidonic acid, but evidence suggests that the majority of arachidonic acid released on the stimulation of cells comes from phospholipids. Flower and Blackwell (1976) demonstrated the uptake of labelled arachidonic acid into cellular lipids on incubation with guinea-pig platelets or spleen slices. Mechanical agitation or immunological challenge of the platelets or spleen tissue resulted in loss of radioactivity from the phospholipid pool only, with a concommittant rise in the levels of free labelled arachidonic acid and increased labelled prostaglandin formation.

The bulk of arachidonate in mammalian cells is esterified almost exclusively in the 2-acyl position in the fatty acyl chains of glycerophospholipids. This would suggest that the enzyme responsible for arachidonic acid release from phospholipids is phospholipase (PL) A_2. PLA_2 perfused into guinea-pig lung (Babilli and Vogt, 1965; Vogt et al., 1969) or frog intestine (Bartels, Vogt and Wille, 1968; Bartels, Kunze, Vogt and Wille, 1970) leads to the appearance of increased amounts of prostaglandins in the perfusate. PLA_2 stimulates the synthesis of prostaglandins in homogenates of human pregnant myometrium (Sykes, Williams and Rogers, 1975). Arachidonic acid also increases prostaglandin synthesis in the frog intestine

(Kunze, 1970) and in pregnant human myometrium and endometrium (Sykes et al., 1975) indicating that the converting enzyme system is highly active in intact tissue but is limited by the amount of substrate available.

PLA_2 in plasma membranes and microsomes is absolutely Ca^{2+}-dependent (Newkirk and Waite, 1973), whereas other lipolytic enzymes do not require Ca^{2+} and are therefore not inhibited by ethylene diamine tetra-acetic acid (EDTA) (McMurray and Magee, 1972). The overall prostaglandin biosynthesis in homogenates of bovine seminal vesicles was markedly stimulated by Ca^{2+} and inhibited by EDTA (Kunze, Bohn and Vogt, 1974) providing further evidence for the role of PLA_2 in prostaglandin formation. It seems, therefore, that the activity of PLA_2 must be an important factor in the biosynthesis of prostaglandins. This thesis aims to identify and isolate factors which either enhance or inhibit the activity of PLA_2, with particular reference to the release of prostaglandin (PG)$F_{2\alpha}$ from the guinea-pig uterus in relation to luteolysis.

1:1 UTERINE $PGF_{2\alpha}$ RELEASE AND LUTEOLYSIS IN THE NON-PREGNANT ANIMAL

The influence of the uterus on the life-span of the corpus luteum was first demonstrated in guinea-pigs when hysterectomy was seen to maintain corpus luteum function long after the normal degeneration time of 14 to 15 days after ovulation (Loeb, 1923). Maturation of follicles was unaffected by the absence of the uterus and removal of the unregressed corpus luteum resulted in a new ovulation, which led Loeb (1927) to conclude "that it is merely the persistence of the corpus luteum which leads to suspension of cyclic changes in the

hysterectomised guinea-pig". The existence of a luteolytic factor secreted from the guinea-pig uterus was proposed (Loeb, 1927).

The prolongation of corpus luteum life-span after hysterectomy was demonstrated in other species, including the cow and sheep (Wiltbank and Casida, 1956), pig (Spies, Zimmerman, Self and Casida, 1958), horse (Ginther and First, 1971) and the pseudo-pregnant rat (Bradbury, 1937), rabbit (Asdell and Hammond, 1933), mouse (Critser, Rutledge and French, 1980) and hamster (Caldwell, Mazer and Wright, 1967). However, ovarian cyclicity was unaffected by removal of the uterus in the dog (Cheval, 1934), ferret (Deanesly and Parkes, 1933), squirrel (Drips, 1919), monkey (Burford and Diddle, 1936), opossum (Hartman, 1925) and phalanger (Clark and Sharman, 1965). In addition, hysterectomy or congenital absence of the uterus, Fallopian tubes and vagina does not prolong the life-span of the corpus luteum in the human (Whitelaw, 1958; Jones and Telinde, 1961; Brown and Matthew, 1962; Beavis, Brown and Smith, 1969; Beling, Marcus and Markham, 1970; Doyle, Barclay, Duncan and Kirton, 1971).

The local action of the luteolytic factor in the guinea-pig was demonstrated by unilateral hysterectomy. The corpora lutea were maintained on the operated side but regressed as normal on the unoperated side (Fischer, 1965; Bland and Donovan, 1966). In sheep, the removal of the uterine horn ipsilateral to the ovary containing the corpus luteum resulted in extension of the oestrous cycle but the removal of the contralateral horn did not (Moor and Rowson, 1966a).

Oxytocin treatment from Days 3-8 shortened oestrous cycles in intact heifers, and in unilaterally hysterectomised heifers in which the retained horn was next to the ovary containing the corpus luteum but cycles remained lengthened in unilaterally hysterectomised

heifers with the retained horn contralateral to the corpus luteum (Ginther, Woody, Mahajan, Janakiraman and Casida, 1967). In the rat, unilateral ovariectomy and unilateral hysterectomy when combined ipsilaterally did not lengthen pseudopregnancy but, when combined contralaterally, pseudopregnancy was prolonged (Barley, Butcher and Inskeep, 1966). These results suggested that the influence of the luteolytic factor from each uterine horn was limited to the ipsilateral ovary. The exceptions are the rabbit, pig and horse where bilateral corpus luteum regression occurs after hemi-hysterectomy (Hunter and Casida, 1967; Anderson, Butcher and Melampy, 1961; Ginther and First, 1971) suggesting that the luteolytic factor may be acting systemically in these species. Indeed, in the pig all but one quarter of one horn needs to be removed before luteal regression is prevented in the contralateral ovary (Du Mesnil du Buisson, 1961).

Pharriss and Wyndgarden (1969) suggested that luteolysis might be induced by a reduction in ovarian blood flow caused by a venoconstricting substance secreted from the uterus. They found that $PGF_{2\alpha}$, a potent venoconstrictor (Ducharme, Weeks and Montgomery, 1968) was luteolytic when infused into pseudopregnant rats (Pharriss and Wyndgarden, 1969). $PGF_{2\alpha}$ also shortened the lifespan of the corpus luteum in the guinea-pig (Blatchley and Donovan, 1969), rabbit (Gutknecht, Cornette and Pharriss, 1969; Gutknecht, Duncan and Wyndgarden, 1972), ewe (McCracken, Glew and Scaramuzzi, 1970; Chamley, Buckmaster, Cain, Cerini, Cerini, Cumming and Goding, 1972), monkey (Kirton, Pharriss and Forbes, 1972), cow (Louis, Hafs and Morrow, 1972; Rowson, Tervit and Brand, 1972), mare (Douglas and Ginther, 1972; Noden, Oxender and Hafs, 1974), sow (Gleeson, 1974; Guthrie and Polge, 1976; Lindloff, Holtz, Elsaesser, Kreikenbaum and

Smidt, 1976; Moeljono, Bazer and Thatcher, 1976), goat (Ott, Nelson and Hixon, 1980), hamster (Labhsetwar, 1971), mouse (Labhsetwar, 1972), rat (Chatterjee, 1973) and Mongolian gerbil (Chaichereon, Meckley and Ginther, 1974). Although 25mg of $PGF_{2\alpha}$ infused intravenously into women on Day 21 of the menstrual cycle led to a sharp decline in progesterone levels in one study (Lehmann, Peters, Breckwoldt and Bettendorf, 1972), other workers found that infusion of $PGF_{2\alpha}$ did not affect the length of the luteal phase or the pattern of steroid secretion in cycling women (Lemaire and Shapiro, 1972; Jewelewicz, Cantor, Dyrenfurth, Warren and Vande Wiele, 1972) or in pregnant women prior to abortion (Speroff; Caldwell, Brock, Anderson and Hobbins, 1972). Also $PGF_{2\alpha}$ was found not to be luteolytic in the bitch (Jochle, Tomlinson and Andersen, 1973) or the ferret (Blatchley and Donovan, 1973).

Distension of the guinea-pig uterus by the introduction of glass beads resulted in shortening of the oestrous cycle (Donovan and Traczyk, 1962; Bland and Donovan, 1968). Distended guinea-pig uterine horns were found to release greater amounts of $PGF_{2\alpha}$ than control non-distended horns (Poyser, Horton, Thompson and Los, 1970; 1971). This demonstrated that $PGF_{2\alpha}$ release from the guinea-pig uterus could be caused by a stimulus known to induce luteolysis. Similarly in sheep, there were shorter cycles and smaller corpora lutea at autopsy in ewes when the corpus luteum was in an ovary adjacent to a uterine horn distended with a coil (IUD)(Ginther, Pope and Casida, 1966) and ewes with an IUD exhibited increased endometrial $PGF_{2\alpha}$ levels in the proximity of the device (Spilman and Duby, 1972). Peripheral plasma levels of PGF were increased in rats and hamsters with IUDs in their uterine horns (Saksena, Lau and Castracane, 1973).

However, for $PGF_{2\alpha}$ to be accepted as the uterine luteolytic factor it had to be shown to be present in uterine venous blood prior to the time of normal luteal regression. $PGF_{2\alpha}$ was identified in sheep uterine venous blood on Day 14, 15 and 16 of the oestrous cycle (Bland, Horton and Poyser, 1971) in concentrations previously found to be capable of reducing progesterone levels (McCracken et al., 1970). $PGF_{2\alpha}$ was not detectable in peripheral venous blood or before Day 4 of the cycle in uterine venous blood, although this may have been due to the insensitivity of the rat fundus strip used as the assay preparation. Increases in $PGF_{2\alpha}$ concentration in uterine venous blood of sheep, coinciding with falling levels of progesterone prior to luteolysis, were confirmed by other workers (McCracken, Carlson, Glew, Goding, Baird, Green and Samuelsson, 1972; Thorburn, Cox, Currie, Restall and Schneider, 1972; Fitzpatrick and Sharma, 1973). $PGF_{2\alpha}$ was also found in the utero-ovarian vein of guinea-pigs at higher concentrations from Day 11 than previously in the cycle, with a peak occurring on Day 15 concurrent with a decrease in progesterone levels (Blatchley, Donovan, Horton and Poyser, 1972; Earthy, Bishop and Flack, 1975). Increased output of $PGF_{2\alpha}$ from the uterus at the time of decreasing progesterone concentration was also seen in the sow (Gleeson and Thorburn, 1973; Gleeson, Thorburn and Cox, 1974; Moeljono, Thatcher, Bazer. Frank, Owens and Wilcox, 1977; Hunter and Poyser, 1982), goat (Homeida and Cooke, 1982) and cow (Nancarrow, Buckmaster, Chamley, Cox, Cumming, Cummins, Drinan, Findlay, Goding, Restall, Schneider and Thorburn, 1973; Shemesh and Hansel, 1975). The accelerated decrease in peripheral plasma progesterone concentrations on Day 17 in pseudopregnant rabbits was found to be associated with an increase in $PGF_{2\alpha}$ levels in uterine venous

plasma (Lytton and Poyser, 1982a), although other workers failed to find a significant increase in $PGF_{2\alpha}$ levels in the rabbit at this time (Carlson and Cole, 1978). However, the latter measurements were made from blood taken from the posterior vena cava and not the uterine vein. $PGF_{2\alpha}$ in uterine venous plasma of pseudopregnant rats increased from Day 4 to Day 6 and again on Day 9 of the cycle (Weems, 1979).

The local nature of the luteolytic action of $PGF_{2\alpha}$

In the sheep, a dose level of $PGF_{2\alpha}$ which was luteolytic when given directly into the ovary had no effect on progesterone secretion when given systemically (McCracken, 1971). $PGF_{2\alpha}$ infused into cyclic heifers on Day 9 resulted in an average cycle length of 11.4 days when infused into the horn ipsilateral to the ovary bearing the corpus luteum and 15.2 days when infused into the horn contralateral to the ovary bearing the corpus luteum (Liehr, Marion and Olson, 1972). Therefore, in both of these species $PGF_{2\alpha}$ is more active when given locally. This is due to the fact that $PGF_{2\alpha}$ is rapidly metabolised in the peripheral circulation (Ferreira and Vane, 1967). However, in the mare the local (intrauterine or intraluteal) administration of $PGF_{2\alpha}$ did not improve the luteolytic efficacy of $PGF_{2\alpha}$ over systemic (intramuscular) administration (Douglas and Ginther, 1975).

Transfer of $PGF_{2\alpha}$ from the uterus to the ovary

For uterine $PGF_{2\alpha}$ to exert a luteolytic effect on the corpus luteum, there must be a method by which $PGF_{2\alpha}$ can be transported from the uterus to the ovary. Selective severance of the tissues between the ovary and the uterus showed that an intact vascular

supply was necessary for corpus luteum regression in the guinea-pig whereas interruption of the oviduct was without effect on cycle length (Fischer, 1967; Oxenreider and Day, 1967). A similar effect was found in the rat (Clemens, Minaguchi and Meites, 1968; Butcher, Barley and Inskeep, 1969), hamster (Orsini, 1968) and sheep (Inskeep and Butcher, 1966; Kiracofe, Menzies, Gier and Spies, 1966). In sheep, autotransplantation of either the ovary (Goding, McCracken and Baird, 1967) or the uterus (Goding, Harrison, Heap and Linzell, 1967) to the neck extended the oestrous cycle, whereas transplantation of both the uterus and the ovaries to the neck had no effect on the time of luteal regression (Harrison, Heap and Linzell, 1968) demonstrating that the vascular continuity of these two organs was necessary for normal luteal regression. Separation of the ovarian artery and uterine vein in sheep resulted in the persistence of the corpus luteum (Barrett, Blockey, Brown, Cumming, Goding, Mole and Obst, 1971) and it was noticed that, not only had the middle uterine vein to be ligated and divided to maintain the corpus luteum, but also any veins anastomosing to the ovarian artery (Baird and Land, 1973). From this evidence, a counter-current mechanism was postulated for $PGF_{2\alpha}$ transference from the uterine vein to the ovarian artery. Labelled $PGF_{2\alpha}$ infused into the uterine vein of the sheep resulted in increased radioactivity in ovarian arterial blood compared with peripheral arterial samples (McCracken et al., 1972), and it was estimated that >2% of the total radioactivity infused into the uterine vein was transferred directly into the ovarian artery.

Although angiographic studies failed to find any "special arrangement" for counter-current exchange in the guinea-pig (Egund and Carter, 1974), previous workers found that arteries supplying

the ovary were in close apposition to, and frequently coiled around the veins which drain the guinea-pig, rat and hamster uteri (Del Campo and Ginther, 1972). Similarly in the sheep and cow, the ovarian artery was seen to take a "tortuous" path over the surface of the utero-ovarian vein (Del Campo and Ginther, 1973). Thus both anatomical and surgical investigations suggested that the counter-current transfer of $PGF_{2\alpha}$ between the utero-ovarian vein and the ovarian artery was the mechanism by which uterine $PGF_{2\alpha}$ reached the ovary.

Other workers have disputed both that $PGF_{2\alpha}$ is luteolytic in sheep (Coudert, Phillips, Palmer and Faiman, 1972) or that the counter-current mechanism exists (Coudert, Phillips, Faiman, Chernecki and Palmer, 1974), although their experimental techniques have been severely criticised in a detailed review of the evidence for $PGF_{2\alpha}$ as the luteolytic hormone in the sheep (Goding, 1974). However, section of the ovarian artery distal to the region where the counter-current transfer of $PGF_{2\alpha}$ is believed to take place, did not interrupt the oestrous cycle in ewes (Lamond and Drost, 1973), and it was suggested that counter-current transfer between the utero-ovarian vein and the ovarian artery could not be the only mechanism for $PGF_{2\alpha}$ transfer from the uterus to the ovary.

Alawachi, Bland and Poyser (1981) proposed that there may be an additional pathway for $PGF_{2\alpha}$ transfer to the ovary via the venous drainage passing alongside the oviduct, as $PGF_{2\alpha}$ levels are higher in the uterine and the oviducal vein than peripheral venous plasma levels between Days 12 and 16 of the sheep oestrous cycle. Increased amounts of $PGF_{2\alpha}$ are also present in uterine lymph from Day 10 of the cycle onwards in non-pregnant sheep (Abdel Rahim, Bland and Poyser, 1983; 1984a). In addition, the insertion of a glass canula

into the uterine branch of the utero-ovarian vein, followed by the disconnection of all other tissues between the uterus and the ovary was followed by a prolongation of luteal function (Abdel Rahim, Bland and Poyser, 1984b), indicating that a pathway involving uterine venous blood alone is insufficient to explain the transfer of $PGF_{2\alpha}$ from the uterus to the ipsilateral ovary.

Although the competence of multiple bicuspid valves in ovarian and uterine lymphatics prevents the retrograde flow of lymph between the uterus and the ovaries (Staples, Fleet and Heap, 1982), it was suggested that the close apposition of the uterine lymphatic vessels to the ovarian artery in the region of the utero-ovarian vein could provide for counter-current transfer of $PGF_{2\alpha}$ from the uterine lymph to the ovarian arterial blood (Abdel Rahim and Bland, 1985). Indeed, injection of $[^3H]$-$PGF_{2\alpha}$ into the uterine lumen of non-pregnant sheep 7-15 days after oestrus resulted in a peak of radioactivity in uterine lymph within 50 minutes (Heap, Fleet and Hamon, 1985). Infusion of $[^3H]$-$PGF_{2\alpha}$ into a uterine lymphatic vessel resulted in a concentration of radioactivity which was higher in plasma from the adjacent utero-ovarian and ovarian veins than in carotid arterial plasma. The local nature of the transfer was indicated by the higher concentration of $[^3H]$-labelled compounds in the ovary and corpus luteum adjacent to the site of intra-lymphatic infusion compared with those in the opposite organs. Intra-lymphatic rather than intra-uterine vein infusion resulted in greater concentrations of $[^3H]$-labelled compounds in the adjacent ovary and corpus luteum suggesting that lymph may play the more important role in $PGF_{2\alpha}$ transferral from the uterus to the ovary in sheep. Selective severance experiments carried out by Bland and Donovan (1969a) in the guinea-pig indicated that the luteolytic

influence of the uterus was probably mediated by the uterine artery or vein but they noted that these vessels had lymph vessels closely associated with them and therefore they could not rule out lymph as a possible route for transferral of the uterine luteolytic hormone from the uterus to the ovary in this species. Thus it seems that the transfer of uterine $PGF_{2\alpha}$ to the uterus may be mediated by a combination of two counter-current mechanisms, one involving uterine venous blood and the other involving uterine lymph with the importance of each mechanism varying between species.

In the rabbit (Del Campo and Ginther, 1972) and horse (Del Campo and Ginther, 1973), extremely limited contact was found between the uterine vein and the ovarian artery. A local action of the luteolytic factor from the uterus has not been proved in either the horse or the rabbit, making a counter-current mechanism for $PGF_{2\alpha}$ transferral from the uterus to the corpus luteum unlikely in these species.

In the pig, the ovarian artery is located between two of the three channels of the utero-ovarian vein (Ginther, 1974), indicating that the vascular anatomy of the pig is compatible with a local veno-arterial pathway. However, autotransplantation of the left ovary to the right side of the abdominal cavity and removal of the right ovary did not disrupt oestrous cycle length in the pig (Harrison and Heap, 1972), and $PGF_{2\alpha}$ infused into one anterior uterine vein on Day 14 of the pig oestrous cycle led to a decrease in progesterone concentrations in both the ipsilateral and contralateral utero-ovarian veins. These findings suggest that $PGF_{2\alpha}$ acts systemically in this species. However, infusion of $PGF_{2\alpha}$ into the jugular vein of the sow did not cause luteolysis (Kotwica, 1980), and it was suggested that lymph is the means by

which $PGF_{2\alpha}$ is transported within the female reproductive system of pig in view of the rich lymphatic vascularization in the pig uterus.

Action of indomethacin and $PGF_{2\alpha}$ antibodies on luteal regression

The evidence for $PGF_{2\alpha}$ as the luteolytic hormone in guinea-pigs was called into doubt when large doses of indomethacin, an aspirin-like drug known to inhibit prostaglandin synthesis (Vane, 1971) and production from guinea-pig uterine homogenates (Poyser, 1972), given orally or subcutaneously failed to lengthen oestrous cycles by more than 3 to 4 days (Marley, 1972; Horton and Poyser, 1973). However, when indomethacin was implanted in the uterus in a slow-release preparation, extension of oestrous cycle length approached that produced by hysterectomy, and the elevated plasma progesterone levels indicated functional corpora lutea (Horton and Poyser, 1973; Marley, 1973). Indomethacin is therefore more effective in the guinea-pig when given locally.

In rabbits, subcutaneous administration of indomethacin extended the length of pseudopregnancy as much as hysterectomy (O'Grady, Caldwell, Auletta and Speroff, 1972). Pseudopregnant mice treated with subcutaneous indomethacin on Days 5-15 or 7-17 had longer interoestrous intervals than control mice or mice treated on Days 9-19 (Critser, Rutledge and French, 1981). Indomethacin or meclofenamic acid also prolonged the interoestrous interval in hysterectomised mice compared to untreated hysterectomised mice, suggesting that extra-uterine $PGF_{2\alpha}$ may also be involved in luteolysis in mice. In rats, a lengthened pseudopregnancy occurred when subcutaneous indomethacin was administered on Days 5 and 6 or 7 and 8 but not when it was given on Days 9 and 10 (Lau, Saksena and

Chang, 1975). Daily indomethacin treatment prolonged pseudopregnancy in hamsters (Lau et al., 1975).

Indomethacin blocked the luteolytic action of oestradiol in normally cycling Rhesus monkeys (Auletta, Caldwell and Speroff, 1976; Auletta, Agins and Scommegna, 1978). Indomethacin alone was associated with a significant increase in peripheral plasma progesterone levels (Auletta et al., 1978) suggesting that prostaglandins are important in physiological luteal regression in monkeys. However, a previous report (Manaugh and Novy, 1976) failed to find any effect on the duration of the luteal phase or on luteal phase plasma progesterone levels measured daily in Rhesus monkeys given oral or subcutaneous indomethacin during the luteal phase of the menstrual cycle.

Subcutaneous indomethacin treatment delayed luteolysis and suppressed the pulsatile appearance of the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), in the peripheral circulation of the goat (Cooke and Homeida, 1985). Subcutaneous indomethacin treatment during the luteal phase of the oestrous cycle resulted in prolonged corpus luteum maintenance in the pig as judged by ovarian morphology on Day 25, serum progesterone concentrations and failure to display oestrus by Day 25 (Kraeling, Rampacek and Kiser, 1981). Indomethacin had no effect on luteal function in hysterectomised gilts indicating that the source of luteolytic $PGF_{2\alpha}$ in the pig is the uterus.

Intrauterine infusions of indomethacin have been demonstrated to increase oestrous cycle length (Lewis and Warren, 1975, 1977a) and to inhibit oestrogen-induced luteolysis (Lewis and Warren, 1974, 1977b) in sheep and cows. However, orally or parenterally administered aspirin, flufenamic acid or 1-p-chloro-benzylidene-

2-methyl-5-methoxy-3-indenylacetic acid (PGH synthetase inhibitors) failed to prolong the oestrous cycle in ewes (Mellin and Busch, 1976), and it appeared that, for PG synthetase inhibitors to be effective in the sheep, they must be administered locally at the uterine level. These results are in conflict with a previous report in which the increase in uterine vein $PGF_{2\alpha}$ levels in ewes with an IUD was abolished by subcutaneous indomethacin treatment (Spilman and Duby, 1972). Indeed, Kann and Lacroix (1982) found that subcutaneous indomethacin administration prevented corpora lutea regression in 3 out of 5 ewes treated from Day 7 in the cycle. Autopsy after slaughter at Day 23 revealed normal corpora lutea, as confirmed by progesterone determination in peripheral blood. Thus systemic administration of indomethacin is able to prevent luteolysis in the ewe.

Active immunisation against $PGF_{2\alpha}$ resulted in elongated oestrous cycles in guinea-pigs (Horton and Poyser, 1974; Hildebrandt-Stark, Marcus, Yoshinaga, Behrman and Greep, 1975). Plasma progesterone levels indicated that corpora lutea were maintained in a functional secretory state after immunisation (Poyser and Horton, 1975) and a good correlation was found between extension of the oestrous cycle and the titre of antibody to $PGF_{2\alpha}$ found in individual guinea-pigs (Horton and Poyser, 1974). Sheep actively immunised against $PGF_{2\alpha}$ also exhibited lengthened cycles (Scaramuzzi, Baird, Wheeler and Land, 1973). Cows passively immunised against $PGF_{2\alpha}$ on Day 16 and sheep passively immunised on Days 13-15 showed evidence of persistent corpora lutea as indicated by plasma progesterone concentrations and the failure of the animals to return to oestrus at least 29 days after treatment (Fairclough, Smith and McGowan, 1981). Post-partum cows, which often exhibit short-lived corpora

lutea, had the lifespans and progesterone secretion of these corpora lutea maintained for >39 days after active immunization against $PGF_{2\alpha}$ (Copelin, Smith, Keisler and Garverick, 1989).

The hormonal stimulus for uterine $PGF_{2\alpha}$ synthesis

The effects of exogenous ovarian steriods on uterine $PGF_{2\alpha}$ output were examined to find the stimulus for the increase in $PGF_{2\alpha}$ concentrations in utero-ovarian venous blood towards the end of the oestrous cycle. Oestrogen treatment of guinea-pigs over Days 4-6 of the cycle resulted in increased $PGF_{2\alpha}$ concentrations in utero-ovarian blood on Day 7 (Blatchley et al., 1972). This effect was abolished in hysterectomised animals indicating that $PGF_{2\alpha}$ in the utero-ovarian vein originates from the uterus and not the ovary.

Ovariectomised guinea-pigs treated with a progesterone implant followed by 3 days of oestradiol injections were found to have the greatest concentration of $PGF_{2\alpha}$ in the utero-ovarian venous blood when compared to ovariectomised guinea-pigs treated with progesterone or oestradiol alone (Blatchley and Poyser, 1974). The output of $PGF_{2\alpha}$ from ovariectomised guinea-pig uteri superfused in vitro was greatest in those guinea-pigs which had undergone progesterone treatment for 7 days followed by 3 days of oestradiol treatment when compared to ovariectomised guinea-pigs which received progesterone or oestradiol alone (Poyser, 1983b).

$PGF_{2\alpha}$ levels in the peripheral plasma of ovariectomised ewes rose after treatment with oestradiol following a sequence of progesterone injections (Caldwell, Tilson, Brock and Speroff, 1972). $PGF_{2\alpha}$ was undetectable in ewes hysterectomised or immunised against oestradiol before treatment. Maximal stimulation of $PGF_{2\alpha}$ secretion from autotransplanted sheep uteri occurred on infusion of

oestradiol after pretreatment with progesterone (Scaramuzzi, Boyle, Wheeler, Land and Baird, 1974). The concentration of $PGF_{2\alpha}$ in uterine tissue of ovariectomised mice showed a highly significant increase after three days of treatment with progesterone followed by three days of oestrogen when compared to either treatment alone (Saksena and Lau, 1973). Progesterone-priming followed by oestradiol treatment was therefore found to be the best exogenous stimulus of increased $PGF_{2\alpha}$ output from the uterus in several species, but the actual physiological stimulus was determined by analysing the concentrations of ovarian steroids present prior to luteolysis. Progesterone levels in most animals increase at the beginning of the cycle and remain elevated until prior to oestrus. Therefore, the variation in plasma oestradiol levels throughout the oestrous cycle was measured in relation to the increase in uterine $PGF_{2\alpha}$ output prior to luteal regression.

In guinea-pig ovarian vein blood, the oestradiol concentration began to rise on Days 9-10 (Joshi, Watson and Labhsetwar, 1973) preceding the increase in $PGF_{2\alpha}$ levels which occurred on Day 11 (Blatchley et al., 1972; Earthy et al., 1975). In the sheep increased amounts of ovarian oestradiol were secreted around Days 10-12 (Cox, Thorburn, Currie and Restall, 1974) which is prior to the first peak of $PGF_{2\alpha}$ on Day 13 (Thorburn, Cox, Currie, Restall and Schnieder, 1973). The peak of oestradiol in the jugular venous plasma of the cow occurs on Days 10-12 (Shemesh, Ayalon and Lindner, 1972) which is before the $PGF_{2\alpha}$ concentration in utero-ovarian blood increases (Nancarrow et al., 1973). Pig peripheral plasma oestradiol levels increase on Days 12-13 (Henricks, Guthrie and Handlin, 1972) which is coincident with increased $PGF_{2\alpha}$ output into utero-ovarian blood (Gleeson and Thorburn, 1973; Gleeson et

al., 1974). Peripheral blood levels of oestradiol increased from Day 10 in the pseudopregnant rat (Welschen, Osman, Dullaart, de Greef, Ulienbroek and de Jong, 1975). In the pseudopregnant hamster, oestradiol in the ovarian venous plasma reached a peak on Days 8 and 9 while PGF in peripheral plasma increased on Day 9 (Shaikh, Birchall and Saksena, 1973). It would appear that increasing oestradiol concentrations at a time when progesterone levels have been elevated for several days, do precede increased $PGF_{2\alpha}$ release from the uterus in vivo and, therefore, oestradiol acting on a progesterone-primed uterus is the physiological stimulus for $PGF_{2\alpha}$ output prior to luteolysis.

In support of this theory, oestrogen was found to be luteolytic in Day-7 guinea-pigs after subcutaneous administration (Choudary and Greenwald, 1968). This effect was abolished by hysterectomy (Bland and Donovan, 1970) indicating that the uterus was involved in mediating the response. Similarly, the injection of oestradiol on Day 10 and Day 11 of the oestrous cycle of the ewe caused early luteal regression but failed to cause regression of the corpora lutea in ewes hysterectomised on Day 5 (Stormshak, Kelley and Hawk, 1969). Indeed, luteal regression could not be induced in hysterectomised ewes in any trial with exogenous oestrogen, indicating that oestrogen-induced corpus luteum regression in sheep requires the presence of the uterus (Bolt and Hawk, 1972). Daily oestrone sulphate treatment from Day 10 of the cow oestrous cycle had a significant luteolytic effect as compared to corn oil-treated controls (Eley, Thatcher and Bazer, 1979). Oestrogen treatment reduced corpus luteum weights in cycling, pregnant and hysterectomised heifers but progesterone levels were consistently higher in the hysterectomised heifers compared to the cycling and

pregnant animals (Kaltenbach, Niswender, Zimmerman and Wiltbank, 1964). Thus, the luteolytic effect of oestrogen is mediated via the uterus.

A phase of follicular growth has been shown to occur in sheep at the same time as oestradiol levels increase (Smeaton and Robertson, 1971) and the destruction of ovarian follicles by X-ray irradation (Karsch, Noveroske, Roche, Norton and Nablandov, 1970) or mechanically (Ginther, 1970) prevented normal luteal regression in sheep and cows (Villa-Godoy, Ireland, Wortman, Ames, Hughes and Fogwell, 1985). These results support the theory that the release of ovarian oestrogen acting on a progesterone-primed uterus is the actual physiological stimulus for increased uterine $PGF_{2\alpha}$ production at the end of the oestrous cycle.

Oxytocin and uterine $PGF_{2\alpha}$ release

The release of uterine $PGF_{2\alpha}$ caused by oestradiol acting on a progesterone-primed uterus is also dependent upon oxytocin in some species. Oxytocin shortened oestrous cycle length in cyclic heifers when injected during the first week post-oestrus (Auletta, Currie and Black, 1972; Anderson and Bowerman, 1963), an effect which was dependent on an intact uterus (Armstrong and Hansel, 1959; Anderson, Bowerman and Melampy, 1965). The luteolytic effect of oxytocin was therefore dependent on a secretion from the uterus. Unilaterally hysterectomised heifers exhibited shortened oestrous cycles in response to oxytocin only if the remaining uterine horn was adjacent to the ovary containing the corpus luteum ovary and not if it were contralateral to the corpus luteum (Ginther et al., 1967), suggesting the involvement of a local utero-ovarian mechanism Oxytocin increased uterine $PGF_{2\alpha}$ output and depressed jugular vein

progesterone levels in heifers treated early in the cycle (Newcomb, Booth and Rowson, 1977; Milvae and Hansel, 1980a). Thus the luteolytic action of oxytocin was mediated through an effect on $PGF_{2\alpha}$ release from the uterus.

Intra-arterial infusion of oxytocin into the sheep uterus had essentially no effect on $PGF_{2\alpha}$ concentration in uterine venous plasma early in the cycle but caused a very pronounced increase in $PGF_{2\alpha}$ secretion late in the luteal phase around Day 14 (Wilson, Roberts and McCracken, 1974; Roberts, Barcikowski, Wilson, Skarnes and McCracken, 1975). This increase in the $PGF_{2\alpha}$-releasing response to oxytocin of the sheep uterus at Day 14 was unrelated to myometrial activity (Roberts and McCracken, 1976). Oxytocin dramatically increased $PGF_{2\alpha}$ concentrations in the posterior vena cava of anoestrous ewes if they had been oestrogen-primed but had negligible effect in untreated ewes (Sharma and Fitzpatrick, 1974). Thus the $PGF_{2\alpha}$ releasing effect of oxytocin in the ewe is dependent on the presence of oestrogen. The number of high affinity binding sites for oxytocin in sheep myometrium and endometrium rose throughout the cycle reaching a peak at oestrus in both tissues, although the binding capacity of the endometrium was twice that of the myometrium at oestrus (Roberts, McCracken, Gavagan and Soloff, 1976). The concentration of uterine oxytocin receptors in cyclic heifers increased around the period of luteolysis and at oestrus, rising on Day 15 in the endometrium and on Day 17 in the myometrium (Jenner, Parkinson and Lamming, 1991). As the concentration of oxytocin receptors in the sheep endometrium changes during the oestrous cycle in parallel with the cyclical variation in the ability of oxytocin to evoke uterine $PGF_{2\alpha}$ secretion, McCracken (1980) proposed that increasing oestradiol levels might regulate

endometrial $PGF_{2\alpha}$ release by controlling the availability of oxytocin receptors.

Similar oestrogen-related responses to oxytocin were found in other species. The sensitivity of the rat uterus to contractions caused by oxytocin *in vitro* increased towards oestrus (Chan, O'Connell, and Pomeroy, 1963) and after administration of oestrogen (Follet and Bentley, 1964). The rat uterus bound oxytocin with high affinity (Soloff and Swartz, 1974), and oestrogen treatment increased the number of oxytocin binding sites in the ovariectomised rat uterus as soon as 6h after treatment (Soloff, 1975). In addition, the production of $PGF_{2\alpha}$ from the endometrium of rat uterine horns superfused *in vitro* was increased by oxytocin in oestrogen-treated ovariectomised animals (Campos, Liggins and Seamark, 1980). Endometrial tissue from ovariectomised rabbits pretreated with oestradiol had a significantly increased rate of release of $PGF_{2\alpha}$ when incubated with oxytocin compared to endometrial tissue from control rabbits, from rabbits pretreated with progesterone, or from rabbits pretreated with progesterone together with oestradiol (Small, Gavagan and Roberts, 1978).

Evidence for the physiological role of oxytocin in uterine $PGF_{2\alpha}$ release in ewes was provided by the discovery that immunisation against oxytocin delayed luteal regression (Flint, Mitchell and Sheldrick, 1979; Sheldrick, Mitchell and Flint, 1980; Schams, Prokopp and Barth, 1983). Similarly in goats active immunization against oxytocin prolonged oestrous cycle lengths from 19.4 to 29.1 days and suppressed peripheral plasma PGFM levels (Cooke and Homeida, 1985).

Hormonal control of uterine receptors

No changes in the concentration of PGFM were found in jugular venous blood of ovariectomised ewes treated with oestradiol, progesterone, both steroids together or with neither steroid following injection of oxytocin on Day 5 or Day 10 of steroid treatment. However, concentrations of PGFM in peripheral plasma increased following the injection of oxytocin on Day 15 of steroid treatment in animals receiving progesterone, but with a larger response occurring in those treated with progesterone and oestradiol together (Homanics and Silvia, 1988). Binding of oxytocin to oviductal membranes from prepubertal, anoestrous and pregnant ewes was low, but in anoestrous ewes which had been treated with progesterone and oestrogen it was similar to values measured in ewes at oestrus (Ayad, McGoff and Wathes, 1990). Thus, the hormonal stimuli which maximally stimulate uterine $PGF_{2\alpha}$ release also cause the maximal $PGF_{2\alpha}$-releasing response to oxytocin and the maximum binding of oxytocin to reproductive tissues. These findings also suggested that progesterone has a role to play in the control of oxytocin receptor concentrations in the sheep endometrium.

Progesterone inhibits the replenishment of oestrogen receptors in the immature, oestrogen-treated rat uterus (Hsueh, Peck and Clark, 1976), human endometrium (Tseng and Gurpide, 1975) and in ovariectomised, oestradiol-treated monkeys (Brenner, Resko and West, 1974) and rats (West, Verhage and Brenner, 1976). Progesterone also prevents the oestrogen-stimulated increase of oestrogen receptors in ovine endometrium (Koligian and Stormshak, 1977a). Indeed, oestrogen receptor concentrations in ovine endometrium are lowest during the mid-luteal phase of the oestrous cycle when progesterone concentrations in systemic blood are maximal (Koligian and

Stormshak, 1977b). Progesterone rapidly and selectively down-regulates oestradiol receptors in the hamster (Evans and Leavitt, 1980) and rat uterus (Okulicz, Evans and Leavitt, 1981).

Therefore, McCracken, Schramm and Okulicz (1984) proposed a hypothesis for steroid control of the oxytocin receptor in the endometrium of the ewe, namely progesterone inhibits the endometrial response to oestrogen by inhibiting oestradiol receptors during the mid-luteal phase, and at the end of the cycle the inhibitory effect of progesterone is lost. Rising oestradiol levels then stimulate the formation of endometrial oxytocin receptors allowing circulating oxytocin to bind and stimulate $PGF_{2\alpha}$ release from the uterine endometrium.

Ovariectomised guinea-pigs treated with oestradiol exhibit an increase in uterine progesterone receptor concentration which is prevented by pretreatment with protein or RNA synthesis inhibitors (Vu Hai, Logeat, Warembourg and Milgrom, 1977), but progesterone administered after oestradiol caused a marked decrease in progesterone receptor concentration which was not affected by protein synthesis inhibitors but was due to enhanced receptor inactivation perhaps by promotion of the translocation of the receptor to the cytosol. The loss of the inhibitory effect of progesterone proposed by McCracken et al. (1984) for the sheep uterus may therefore be due to down-regulation of progesterone receptors by progesterone.

However, Vallet, Lamming and Batten, (1990a) found progestagen pre-treatment of ewes followed by ovariectomy and progesterone treatment for 12 days caused a large increase in oxytocin receptors and no further increase occurred when ewes were given oestradiol on Days 11 and 12 or when progesterone was withdrawn on Days 11 and 12,

or--when these two treatments were combined. Oxytocin administration increased plasma PGFM concentrations in ewes which received progestagen pre-treatment followed by ovariectomy and progesterone treatment for 12 days, progesterone treatment for 12 days plus oestradiol on Day 11 and Day 12 of progesterone treatment, progesterone withdrawal on Day 11 and 12, or progesterone withdrawal and oestradiol treatment combined. Thus, long term progesterone treatment alone was sufficient to induce both oxytocin receptors and PGF release in response to oxytocin. They suggested that progesterone played the dominant role in the control of luteolysis, and that oestrogen may modify the time course of PGF release in response to oxytocin and maintain the luteolytic mechanism when progesterone concentrations fall in response to PGF.

Flint, Leat, Sheldrick and Stewart (1986) found that the stimulation of ovine endometrial prostaglandin synthesis by oxytocin was associated with an increase in the hydrolysis of phosphoinositides by activation of phospholipase C (PLC), to give diacylglycerol (DAG) and inositol phosphates (IP). They suggested that DAG forms the source of arachidonic acid for $PGF_{2\alpha}$ synthesis. Second messengers associated with the activation of PLC, such as the calcium ionophore A23187 and phorbol 12-myristate 13-acetate (PMA, an activator of protein kinase C), enhanced release of $PGF_{2\alpha}$ from ovine endometrial tissue (Silvia and Homanics, 1988). Oxytocin increased the formation of inositol triphosphate (IP_3) and total inositol phosphates (IP) in endometrium from Day-16 cyclic ewes but not from Day-12 cyclic ewes (Mirando, Ott, Vallet, Davis and Bazer, 1990a). Thus, the release of uterine $PGF_{2\alpha}$ in the ewe by oxytocin may be due to activation of PLC.

However, there was no change in the content of phosphoinositides

or synthesis of phosphoinositides by the guinea-pig endometrium between Day 7 and Day 15 of the cycle (Ning and Poyser, 1984). PMA had no effect on the output of prostaglandins from Day-7 and Day-15 guinea-pig endometrium maintained in culture (Riley and Poyser, 1987a), or on the output of prostaglandin from Day-7 and Day-15 guinea-pig uterus superfused *in vitro* (Poyser, 1987a). Both PLA_2 and PLC stimulated the outputs of prostaglandin from Day-7 and Day-15 guinea-pig uterus superfused *in vitro*. However, PLA_2 led to a pattern of prostaglandin release similar to that caused by Ca^{2+}, whereas PLC caused much more endogenous arachidonic acid to be transformed into PGE_2 (Poyser, 1987a). As oestradiol acting on a progesterone-primed uterus stimulated very little PGE_2 formation from the guinea-pig uterus (Poyser and Brydon, 1983; Poyser, 1983a), it would suggest that PLA_2 and not PLC is involved in the stimulation of $PGF_{2\alpha}$ output by ovarian steroid hormones. In addition both $PGF_{2\alpha}$ synthesis and arachidonic acid release from guinea-pig uterine homogenates were inhibited by p-bromophenacyl bromide, an inhibitor of PLA_2 (Mitchell, Poyser and Wilson, 1977). However, this effect was not overcome by the addition of exogenous arachidonic acid, indicating that p-bromophenacyl bromide also inhibited the conversion of arachidonic acid into prostaglandins and was not specific for PLA_2.

In contrast to the sheep, goat and cow, oxytocin does not seem to be involved in the release of $PGF_{2\alpha}$ from the uterus of the guinea-pig. Oxytocin injected into guinea-pigs during any part of the cycle failed to initiate premature corpus luteum regression (Donovan, 1961), and oxytocin had no effect on prostaglandin release from the Day-7 or Day-15 guinea-pig uterus superfused *in vitro* (Poyser and Brydon, 1983). In addition, oxytocin did not stimulate

prostaglandin output from guinea-pig endometrium maintained in tissue culture (Riley and Poyser, 1987b), although this contradicts the finding of a previous report (Leaver and Seawright, 1982). Also, oxytocin and oestradiol receptors increased in the guinea-pig myometrium prior to parturition despite the absence of a fall in plasma progesterone levels (Alexandrova and Soloff, 1980).

These results indicate that oxytocin is capable of stimulating uterine release of $PGF_{2\alpha}$ in several species, possibly by an action on PLC, and this may be part of the physiological mechanism responsible for the increase in $PGF_{2\alpha}$ output from the uterus of the sheep and goat at luteolysis as immunization against oxytocin extends oestrous cycle length in these species. However, oxytocin does not affect uterine $PGF_{2\alpha}$ release in the guinea-pig, nor does the enzyme PLC appear to be involved in the increase in $PGF_{2\alpha}$ output from the guinea-pig uterus at the time of luteolysis.

Pulsatile release of $PGF_{2\alpha}$ and it's relationship to oxytocin secretion

Continuous infusion of $PGF_{2\alpha}$ into the arterial supply of the ovary of the sheep revealed that the minimal effective luteolytic dose was in the order of 2μg/h (Goding, Baird, Cumming and McCracken, 1971). However, further studies in the sheep revealed that $PGF_{2\alpha}$ secretion from the ovine uterus at the time of luteolysis is not as a continuous surge but rather as a series of pulses lasting 1h or less (Thorburn et al., 1973; Barcikowski, Carlson, Wilson and McCracken, 1974; Baird, Land, Scaramuzzi and Wheeler, 1976). This phenomenon is also seen in the cow (Nancarrow et al., 1973) and pig (Gleeson and Thorburn, 1973; Gleeson et al., 1974). These intermittent pulses of $PGF_{2\alpha}$ were confirmed by the

measurement of pulses of the primary metabolite of $PGF_{2\alpha}$ (PGFM) in the peripheral blood of sheep (Kindahl, Granstrom, Edqvist and Eneroth, 1976; Peterson, Tervit, Fairclough, Havik and Smith, 1976; Fairclough, Moore, McGowan, Peterson, Smith, Tervit and Watkins, 1980), cows (Kindahl, Lindell and Edqvist, 1981) and goats (Homeida and Cooke, 1982).

Therefore Schramm, Bovaird, Glew, Schramm and McCracken (1983) re-examined the minimal effective luteolytic dose of $PGF_{2\alpha}$ by infusing pulses of $PGF_{2\alpha}$ into the autotransplanted ovary of the sheep at a frequency calculated to mimic physiological exposure of the <u>in situ</u> ovary to $PGF_{2\alpha}$ during naturally occurring luteolysis. They found that four 1h-long pulses of $PGF_{2\alpha}$ in 19h caused complete corpus luteum regression in only 1 of 4 animals whereas the addition of a fifth pulse (5 pulses in 25h) caused permanent regression in 4 out of 4 animals. The average total effective dose of $PGF_{2\alpha}$ required to induce luteal regression when given as five 1h-long pulses was 1/40th of the regarded minimal effective dose when given by constant infusion into the ovarian artery. A more protracted regimen of pulsatile $PGF_{2\alpha}$ infusion given once per day for 4 days failed to cause permanent luteal regression, suggesting that a relatively short pulse frequency over a minimal period of 24h is a necessary condition for physiological regression of the corpus luteum. To date, it has proved impossible to examine the pattern of $PGF_{2\alpha}$ release in smaller animals (e.g. the guinea-pig and rat) due to difficulties in sampling. It is not known whether this pulsatile pattern of $PGF_{2\alpha}$ release is a feature of the control of luteolysis in smaller animals.

The question then arose as to what was the mechanism that controlled the pulsatile release of $PGF_{2\alpha}$. It had previously been

proposed that the pulsatile pattern of uterine $PGF_{2\alpha}$ secretion in sheep at luteolysis might be related to secretion of oxytocin from the posterior pituitary (Roberts and McCracken, 1976), Indeed, a temporal relationship between the levels of neurophysins associated with oxytocin release and PGFM in jugular venous blood of sheep during functional regression of the corpus luteum was demonstrated (Fairclough et al., 1980) and a pulsatile component in levels of oxytocin in peripheral plasma was seen in sheep (Mitchell, Kramer, Brennecke and Webb, 1982) and goats (Homeida and Cooke, 1983). However, peripheral plasma concentrations of oxytocin in cycling ewes were found to be relatively high during the luteal phase of the cycle but fell at oestrus along with progesterone concentrations (Webb, Mitchell, Falconer and Robinson, 1981; Sheldrick and Flint, 1981; Schams, Lahlou-Kassi and Glatzel, 1982), implying that increasing concentrations of circulating oxytocin at the time of luteolysis were unlikely to be the stimulus for increased uterine $PGF_{2\alpha}$ production in the sheep. These results did suggest that the ovary might be the source of circulating oxytocin as the plasma concentrations of oxytocin rose and fell at the same time as the corpus luteum gained and lost its progesterone secretory capabilities. Indeed, oxytocin was found to be present in high concentrations in ovine and bovine corpora lutea (Wathes and Swann, 1982; Fields, Eldridge, Fuchs, Roberts and Fields, 1983), with the large luteal cells being directly responsible for oxytocin synthesis and secretion (Rodgers, O'Shea, Findlay, Flint and Sheldrick, 1983).

The $PGF_{2\alpha}$ analogue cloprostenol stimulated oxytocin secretion into the ovarian vein (Flint and Sheldrick, 1982a, 1982b) and, although oxytocin levels fell at oestrus, frequent sampling revealed that two-thirds of the pulses of uterine secretion of $PGF_{2\alpha}$ in the

sheep were accompanied by rises in peripheral plasma oxytocin levels. Flint and Sheldrick (1983) suggested that the secretion of $PGF_{2\alpha}$ and oxytocin could be linked by a positive feedback loop, which might account for the pulsatile nature of the secretion of both oxytocin and $PGF_{2\alpha}$. Evidence to support this theory came from the demonstration that concentrations of oxytocin in corpora lutea were reduced after hysterectomy in sheep (Sheldrick and Flint, 1983), indicating that oxytocin requires the presence of endogenous uterine $PGF_{2\alpha}$ for it's release. In addition, cloprostenol was unable to raise levels of oxytocin in ovarian and jugular venous plasma in hysterectomised animals. However, administration of cloprostenol to hysterectomised ewes resulted in luteal regression which occurred as rapidly as in intact animals suggesting that oxytocin in the corpus luteum is unlikely to be involved in the intraluteal events mediating prostaglandin induced luteolysis.

A similar positive feedback loop between ovarian oxytocin and uterine $PGF_{2\alpha}$ may exist in the goat. Concentrations of oxytocin in the peripheral plasma of the goat decrease from Day 12 onwards and this decline is characterized by frequent pulses of oxytocin (Homeida and Cooke, 1983). Subcutaneous indomethacin treatment between Days 11 and 16 delayed luteolysis, and suppressed both the decline in oxytocin concentration and the pulsatile appearance of both oxytocin and PGFM in the peripheral circulation of the goat, indicating that $PGF_{2\alpha}$ may be responsible for the pulsatile release of oxytocin at luteolysis in this species (Cooke and Homeida, 1984). In addition, pretreatment with indomethacin prevented the $PGF_{2\alpha}$-induced luteolysis and increases in jugular PGFM and oxytocin in the goat (Homeida and Cooke, 1985). $PGF_{2\alpha}$ injections were without effect on peripheral PGFM and oxytocin concentrations in

ovariectomised goats, suggesting that $PGF_{2\alpha}$ induces the release of ovarian oxytocin in the goat. To account for cessation of each episode of oxytocin and $PGF_{2\alpha}$ secretion, it was suggested that oxytocin might cause down-regulation of oxytocin receptors. Indeed, continuous intravenous infusion of oxytocin between Days 13 and 21 after oestrus in the ewe delayed return to oestrus by 7 days and blocked the rise in uterine oxytocin receptors which normally precedes oestrus (Flint and Sheldrick, 1985). Steroid-primed, ovariectomised ewes treated intravenously with oxytocin at intervals of 1, 2, 4 or 6h exhibited large increases in PGFM in peripheral circulation after the initial dose (Sheldrick and Flint, 1986). However, the subsequent dose failed to elicit as large an increase in circulating PGFM, although the response was larger the longer the period between the two doses. Thus, a period of uterine refractoriness followed administration of oxytocin to progestagen plus oestradiol-primed ovariectomised ewes. However, uterine oxytocin receptor concentrations were unchanged 2h after oxytocin administration suggesting that the refractoriness of the uterus to oxytocin was not due to down-regulation of the oxytocin receptor. The timing of the period of uterine refractoriness following oxytocin administration was consistent with the frequency of the pulses of uterine $PGF_{2\alpha}$ secretion at luteolysis, which have been observed to occur at intervals of greater than 6h and with the frequency with which $PGF_{2\alpha}$ must be administered to obtain enhanced luteolytic potency (Schramm et al., 1983).

Mechanism of steroid action

The mechanism by which steroids, such as progesterone and oestradiol, function is normally determined by binding to a nuclear

receptor. The hormone-receptor complex then interacts with regulatory DNA sequences known as hormone-response elements. Hormone-response elements can either stimulate promoter activity by acting as transcriptional enhancers, or repress transcription by interfering with the activity of other promoter elements. Thus steroid action is mediated through an effect on protein synthesis.

Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, given by intrauterine administration to sheep on Day 11, prevented corpus luteum regression (French and Casida, 1973). Intrauterine but not systemic administration of actinomycin D on Day 10 increased oestrous cycle length in guinea-pigs (Poyser, 1979). Peripheral progesterone levels remained elevated during lengthened cycles and $PGF_{2\alpha}$ production by uterine homogenates *in vitro* on Day 15 was much lower in those guinea-pigs which had received intrauterine actinomycin D than in control guinea-pigs. The *in vitro* output of $PGF_{2\alpha}$ from guinea-pig uterus on Day 15 was reduced by 80-85% after intrauterine administration of actinomycin D on Day 10 (Poyser and Riley, 1987). None of the above effects could be reversed by oestradiol.

Actinomycin D, cycloheximide (an inhibitor of the elongation step of protein transcription) and puromycin (a releaser of nascent polypeptide chains before their synthesis is complete) reduced prostaglandin output from Day-7 and Day-15 guinea-pig endometrium cultured over 24h when they were included in the culture medium (Riley and Poyser, 1989). Prostaglandin output was not reduced when these compounds were superfused over Day-7 and Day-15 guinea-pig uterus for 20 min, indicating that these compounds do not have a rapid inhibitory effect on endometrial prostaglandin synthesis. These protein synthesis inhibitory compounds also reduced the

synthesis of secreted and cellular proteins by Day-7 and Day-15 endometrium in culture, and intrauterine administration of actinomycin D on Day 10 inhibited the synthesis of secreted and cellular proteins by Day-15 endometrium in culture. It would seem, therefore, that the release of $PGF_{2\alpha}$ from guinea-pig endometrium towards the end of the cycle is dependent upon increased endometrial protein synthesis.

Site of action of oestrogen and progesterone

PGH synthase exhibits self-catalysed breakdown (Lands, 1979) so, as prostaglandins are formed, the enzyme catalysing the reaction is inactivated. Consequently the total amount of prostaglandins synthesised by endometrial homogenates is indicative of the concentration of PGH synthase, especially as the metabolism of prostaglandins in the absence of exogenous, nicotinamide-adenine dinucleotide (NAD^+) by the guinea-pig uterus is negligible (<5%) (Poyser, 1979). Naylor and Poyser (1975) demonstrated that oestradiol, but not progesterone, could stimulate an increase in uterine $PGF_{2\alpha}$ synthesising capacity in the guinea-pig. This was confirmed in later experiments in which ovariectomised guinea-pigs were treated *in vivo* with progesterone daily for 7 days followed by oestradiol benzoate for 3 days, or with either steroid treatment alone (Poyser, 1983a). Oestradiol alone caused the maximal increase in $PGF_{2\alpha}$ synthesis by endometrial homogenates, an effect which was reduced by progesterone pretreatment (Poyser, 1983a). However, oestradiol causes only a small release of $PGF_{2\alpha}$ from the guinea-pig uterus superfused *in vitro* and progesterone priming was needed for oestradiol to produce a large release of $PGF_{2\alpha}$ (Poyser, 1983b). Thus, the enzymic capacity of the guinea-pig uterus to

synthesise $PGF_{2\alpha}$ is controlled by oestrogen alone and diminished by progesterone, but the stimulation of the synthesis and release of $PGF_{2\alpha}$ by oestrogen is dependent on progesterone-priming. It would seem, therefore, that changes in endometrial PGH synthetase concentrations are not the direct cause of changes in endometrial $PGF_{2\alpha}$ output at the end of the cycle. In addition, $PGF_{2\alpha}$ output from the guinea-pig uterus superfused in vitro increased 21.9-fold between Day 7 and Day 15 (Poyser and Brydon, 1983) whereas the prostaglandin synthesising capacity of guinea-pig uterine homogenates increased only 2-fold between Day 7 and Day 15 of the cycle (Poyser, 1983a). Therefore, an increase in the supply of precursor arachidonic acid is more likely to be the stimulus of increased uterine $PGF_{2\alpha}$ production at the time of luteolysis rather than an increase in the PGH synthetase concentration.

The stimulation of endometrial phospholipase A_2

Less than 0.1% of arachidonic acid in guinea-pig uterus was found in its unesterified form, and 93% of the bound arachidonic acid was found to be esterified to uterine phospholipids of which 80% was bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Leaver and Poyser, 1981). Studies on arachidonic acid uptake into guinea-pig endometrium in culture showed that more tritiated arachidonic acid, but not oleic acid, was taken up into phospholipids on Day 15 than on Day 7 of the cycle (Ning, Leaver and Poyser, 1983). In addition, on Day 15, but not on Day 7, there was an apparent 50-80% decrease in tritiated arachidonic acid content of PC, PE and triglyceride from cultured guinea-pig endometrium. These results suggest that phospholipids, particularly PC and PE, may be the source of arachidonic acid for the increase in $PGF_{2\alpha}$ from the

uterus at luteolysis.

As the release of arachidonic acid from cellular lipids is the rate-limiting step in prostaglandin formation and as PLC does not appear to be involved in the increase in uterine $PGF_{2\alpha}$ release in the guinea-pig at luteolysis, the activity of PLA_2 is probably the most important factor in increasing guinea-pig uterine $PGF_{2\alpha}$ release. Indeed, 85-95% of PLA_2 present in the guinea-pig endometrium was found in the microsomal fraction and the activity of this membrane-bound PLA_2 increased 1.5- to 1.9-fold between Day 7 and Day 16 of the cycle (Downing and Poyser, 1983). However, calculations showed that the absolute activity of microsomal PLA_2 was more than adequate on both Day 7 and Day 16 of the cycle for sufficient arachidonic acid to be released in order to account for the amounts of $PGF_{2\alpha}$ synthesised by and released from the guinea-pig uterus.

Since microsomal PLA_2 in the guinea-pig endometrium is maximally active in 7mM Ca^{2+} (Downing and Poyser, 1983), which is several fold higher than physiological Ca^{2+} concentrations, a mechanism controlling the activation of PLA_2 by regulating free intracellular Ca^{2+} concentrations is probably of more importance than the absolute activity of microsomal PLA_2 in providing free arachidonic acid for $PGF_{2\alpha}$ synthesis by the endometrium. Indeed, the calcium ionophore A23187 stimulates $PGF_{2\alpha}$ output from cultured Day-7 and Day-15 guinea-pig endometrium (Leaver and Seawright, 1982). The stimulation of $PGF_{2\alpha}$ release from guinea-pig uterus on Day 7 and Day 15 of the cycle caused by the A23187 (Poyser and Brydon, 1983; Poyser, 1983b) was abolished by superfusing the tissue with Krebs' solution lacking Ca^{2+}, although the basal outputs of $PGF_{2\alpha}$ from the Day-15 guinea-pig uterus superfused <u>in vitro</u> were

unaffected by ommitting Ca^{2+} from the Krebs' solution (Poyser, 1984b). TMB-8 (an intracellular Ca^{2+} antagonist), trifluoperazine and W-7 (both calmodulin antagonists) inhibited the increase in output of $PGF_{2\alpha}$ from guinea-pig uterus superfused in vitro caused by A23187 (Poyser, 1985a, 1985b). The output of $PGF_{2\alpha}$ from Day-15 guinea-pig endometrium maintained in culture for 3 days was significantly reduced by the use of Ca^{2+} depleted medium, EGTA (a Ca^{2+} chelator), TMB-8, trifluoperazine and W-7 (Riley and Poyser, 1987a). Therefore, extracellular and intracellular Ca^{2+}, possibly acting via calmodulin, appear to be necessary for the increased output of $PGF_{2\alpha}$ from the guinea-pig uterus after Day 11 of the oestrous cycle.

Phospholipase A_2 and G-proteins

Recent work has shown that the release of arachidonic acid from membrane phospholipids may occur via the receptor activation of phospholipase A_2 mediated by a G-protein. G-proteins are transduction elements which couple the receptor recognition site to the effector molecule. They are heterotrimers consisting of a major subunit (α), of variable molecular weight, and two smaller tightly coupled subunits (β and γ). Agonist binding produces a change in the receptor G-protein interaction promoting the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α subunit. The activated α-GTP subunit then dissociates from the $\beta\gamma$ subunits and one or both of these interacts with the effector molecules. Such effectors include ion channels, and enzymes that generate regulatory molecules or second messengers. Termination of the signal occurs when the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, releasing inorganic phosphate (Pi), and α-GDP recombines

with $\beta\gamma$ to end the cycle. Thus, nonhydrolysable analogues of GTP, such as Gpp(NH)p or GTP-γS, produce persistent activation of α subunits and persistent dissociation of α from $\beta\gamma$. A similar effect is produced by millimolar concentrations of fluoride which confer upon the GDP-bound protein the structural and functional properties of the GTP-bound one, thus activating the G-protein. Fluoride activation of G-proteins was found to require trace amounts of aluminium and it was suggested that AlF_4^- was the active species (Sternweis and Gilman, 1982). Due to the striking structural similarities between AlF_4^- and PO_4^{3-}, it was proposed that AlF_4^- interacts with GDP in the nucleotide site, where it mimics the role of the γ-phosphate of GTP (Bigay, Deterre, Pfister and Chabre, 1985).

The most studied G-proteins are linked to the phototransducing molecule rhodopsin, the muscarinic acetylcholine receptor, and the β-adrenergic receptor, and are known as G_t, G_i and G_s respectively. The bacterial toxins from Vibrio cholera and Bordatella pertussis can covalently modify G-proteins by the addition of an ADP-ribose group to the α subunit. Originally the cholera and pertussis toxin substrates, G_s and G_i, were thought only to stimulate and inhibit adenylate cyclase, respectively, but both α subunits are now known to have a broader function including regulation of ion transport and PI metabolism. G_{olf} which is closely related to G_s, is also an activator of adenylate cyclase and a cholera toxin substrate but is found only in olfactory cilia. G_{t1} and G_{t2}, collectively known as transducin (G_t), are the G-proteins of retinal rod and cone outer segments respectively, which couple light activated rhodopsin to a cyclic GMP phosphodiesterase and are substrates for both toxins. G_o, which is

found in high concentrations in nervous tissue, is a substrate for pertussis toxin ADP-ribosylation like G_i and regulates potassium and calcium channels. The above G-proteins have been both isolated and have a known function (for review see Casperson and Bourne, 1987; Lochrie and Simon, 1988; Allende, 1988; Weiss, Kelleher, Woon, Soparkar, Osawa, Heasley and Johnson, 1988; Chabre and Deterre, 1989; Ross, 1989). However, the genes encoding many other G proteins with unknown functions have been isolated and the involvement of as yet unidentified G proteins in many signalling pathways has been inferred by the effects of guanine nucleotide analogues and bacterial toxins.

In guinea-pig neutrophils, pertussis toxin inhibited the release of arachidonic acid produced by stimulation of the N-formyl peptide receptor but had no effect on arachidonic acid release in response to A23187 (Bokoch and Gillman, 1984). The activity of the toxin was correlated with its ability to catalyse the ADP-ribosylation of a 41kDa membrane protein which co-migrated with the α-subunit of G_i on SDS polyacrylamide gel.

GTP-γS introduced into permeabilized rat thyroid (FRTL5) cells resulted in IP formation and arachidonic acid release (Burch, Luini and Axelrod, 1986). However, the production of arachidonic acid was not related to PLC activity as neomycin (an inhibitor of PLC) inhibited GTP-γS stimulated IP formation but was without effect on GTP-γS stimulated arachidonic acid release. In addition, pertussis toxin inhibited noradrenaline-stimulated arachidonic acid release but not noradrenaline-stimulated inositol phosphate formation. This stimulation of arachidonic acid release was also inhibited by decreased extracellular calcium and by TMB-8, suggesting a role for PLA_2. Light activation of PLA_2 was shown to be a transducin-

dependent mechanism in rod outer segments of bovine retina (Jelsema and Axelrod, 1987). Both light and GTP-γS led to a several fold increase in PLA_2 activity in transducin-rich but not transducin-poor rod outer segments. Pertussis toxin treatment prevented the stimulation of PLA_2 by exogenous transducin in the presence of light. These studies showed that PLA_2 could be activated by agonist binding to a membrane receptor coupled to the enzyme via a pertussis-toxin sensitive G-protein.

The concentration of a 41kDa protein, which was [^{32}P]-ADP ribosylated by pertussis toxin in the presence of [^{32}P]-NAD, in membrane fractions prepared from the uterine endometrium of ovariectomised sheep was increased by progesterone or oestrogen plus progesterone treatment in vivo (Flint, 1988). The presence of a G-protein in sheep uterine endometrium which could be controlled by ovarian steroids provided further evidence that a G-protein may be involved in regulating PLA_2 activity at luteolysis. In addition, pertussis toxin did not affect the increased phosphoinositide hydrolysis in endometrial slices obtained from progesterone plus oestrogen-treated sheep incubated with oxytocin, indicating that this G-protein was not involved in mediating the effects of oxytocin and was therefore probably not linked to PLC.

Summary and aims in the non-pregnant guinea-pig

In summary, the release of luteolytic $PGF_{2\alpha}$ at the end of the cycle in the guinea-pig and several other species of non-primate mammals is caused by oestradiol acting on a progesterone-primed uterus. Oxytocin is alsó necessary in some species (i.e. sheep, cow, goat), but not in the guinea-pig. The actions of oestradiol and progesterone are mediated via an increase in protein synthesis.

PLA_2, the enzyme responsible for the release of $PGF_{2\alpha}$ from the guinea-pig endometrium, requires the mobilisation of calcium for maximum activity. It has been proposed that oestradiol causes the synthesis by the guinea-pig endometrium of a protein ("lipostimulin") which acts in a progesterone-primed uterus to raise the endometrial intracellular free calcium concentration via an influx of extracellular calcium and/or release of intracellular calcium (Poyser, 1984a). Consequently, the production of proteins by the guinea-pig endometrium has been investigated and their effects on the activity of PLA_2 and on $PGF_{2\alpha}$ output from the guinea-pig endometrium examined to see whether these proteins are involved in the stimulation of endometrial $PGF_{2\alpha}$ synthesis. Also, as the activity of PLA_2 is stimulated by a G-protein in several tissues, steroid activation of endometrial PLA_2 may involve a G-protein link. Studies in this thesis have therefore also investigated whether a G-protein is involved in the stimulation of $PGF_{2\alpha}$ synthesis in the guinea-pig endometrium.

1:2 THE RELEASE OF UTERINE LUTEOLYTIC HORMONE DURING EARLY PREGNANCY

Guinea-pigs ovariectomised at Day 21 of gestation aborted unless given progesterone therapy following ovariectomy, whereas ovariectomy after Day 30 of gestation was without effect (Heap and Deanesly, 1966; Csapo, Puri and Tarro, 1981). Thus, the secretion of progesterone from the corpus luteum of the guinea-pig is necessary up to about Day 25 of pregnancy. At this time the "luteo-placental shift" occurs and the placenta takes over progesterone production (Heap and Deanesly, 1964). Consequently, the life-span of the corpus luteum in the pregnant guinea-pig has to be extended in order to continue producing progesterone for the first third of pregnancy. The luteolytic influence of the uterus must therefore be negated and release of $PGF_{2\alpha}$ prevented in pregnant animals.

Indeed the levels of $PGF_{2\alpha}$ measured in the utero-ovarian vein of pregnant guinea-pigs remained low on Day 15 compared to the elevation in $PGF_{2\alpha}$ levels exhibited by non-pregnant animals at this time (Blatchley, Maule Walker and Poyser, 1975a, 1975b; Antonini, Turner and Pauerstein, 1976). This was not due to increased metabolism of $PGF_{2\alpha}$ as metabolism of PGE_2 and $PGF_{2\alpha}$ by homogenates of Day-15 pregnant and Day-15 non-pregnant guinea-pig uterus was found to be only 8-20% (Maule Walker and Poyser, 1978).

Like the guinea-pig, the sheep requires progesterone secretion from the corpus luteum for the first third of pregnancy. The major peaks of $PGF_{2\alpha}$ in utero-ovarian blood found on Day 15 and Day 16 in cyclic ewes were absent in pregnant ewes (Thorburn et al., 1973; Barcikowski et al., 1974; Peterson et al., 1976). Significantly fewer pulses of $PGF_{2\alpha}$, which were also significantly smaller in amplitude, were measured in utero-ovarian venous samples collected from early pregnant sheep compared to non-pregnant sheep on

Days13-16 of the oestrous cycle (Hooper, Watkins and Thorburn, 1986). However, Zarco, Stabenfeldt, Kindahl, Quirke and Granstrom (1984) found that the frequency of PGFM pulses in the jugular vein during the period of expected luteolysis was lower in ewes with a persistent corpus luteum than normal cyclic ewes, whereas the pulse amplitudes were not different. A decrease in the frequency of pulses of luteolytic $PGF_{2\alpha}$ in pregnant animals with no change in the pulse amplitude might explain why Burgess, Ralph, Jenkin and Thorburn, (1990) found that basal concentrations of PGFM were significantly elevated in utero-ovarian venous plasma on Day 14 of pregnancy compared to that observed on Day 14 of the cycle or Days 21-25 of pregnancy. $PGF_{2\alpha}$ concentrations were also low in the pregnant cow (Kindahl, Edqvist, Bane and Granstrom, 1976), pig (Moeljono et al., 1977), horse (Douglas and Ginther, 1976; Kindahl, Knudsen, Madej and Edqvist, 1982; Zavy, Vernon, Asquith, Bazer and Sharp, 1984), goat (Homeida and Cooke, 1982) and rabbit (Lytton and Poyser, 1982a, 1982b).

As oestrogen acting on a progesterone-primed uterus is the stimulus for increased $PGF_{2\alpha}$ concentrations at the end of the oestrous cycle in the guinea-pig, it was suggested that the extended luteal life span observed during pregnancy was due to suppression of oestradiol release (Blatchley et al., 1975b). Oestradiol levels in the utero-ovarian venous plasma were lower in pregnant guinea-pigs than in non-pregnant guinea-pigs on Day 12 and Day 15 (Blatchley et al., 1975a, 1975b). A lack of increase in oestradiol levels was also demonstrated in the pregnant ewe (Cox et al., 1974), cow (Henricks, Dickey, Hill and Johnston, 1972), pig (Guthrie, Henricks and Handlin, 1972) and horse (Kindahl et al., 1982).

However, the effects of exogenous oestradiol tended to disprove

this theory. Oestradiol treatment of guinea-pigs from Days 10-14 of pregnancy resulted in decreased plasma progesterone concentrations and increased uterine $PGF_{2\alpha}$ output and endometrial $PGF_{2\alpha}$ synthesising capacity in only 25% of treated guinea-pigs (Poyser, 1984a). This supported previous work in which oestrogen administered on Day 10 and Day 11 to pregnant guinea-pigs did not affect progesterone levels in 12 out of 16 animals (Evans, Sim, Merrick and Kelleher, 1981). These results indicate that it is not the prevention of the release of oestradiol which is the factor important in maintaining the guinea-pig corpus luteum during pregnancy.

In fact, oestrogen has been proposed to be involved in luteal maintenance as large doses given towards the end of the cycle prolonged luteal function in the non-pregnant guinea-pig (Illingworth, 1969; Illingworth and Perry, 1973), sheep (Piper and Foote, 1965; Denamur, Martinet and Short, 1970), and pig (Gardner, First and Casida, 1963; Chakraborty, England and Stormshak, 1972; Geisert, Zavy, Wetteman and Bigges, 1987). Indeed, oestradiol valerate treatment of the non-pregnant pig on Days 11-15 not only lengthened the oestrous cycle but maintained elevated plasma progesterone levels and reduced $PGF_{2\alpha}$ concentrations in the uterine venous plasma (Frank, Bazer, Thatcher and Wilcox, 1977).

Oestrogen has been proposed to be a major factor in the antiluteolytic mechanism in the pregnant pig as the pig blastocyst has the ability to synthesise and secrete oestrogens from Day 11 or 12 of pregnancy (Perry, Heap and Amoroso, 1973; Perry, Heap and Burton, 1975; Gadsby, Heap and Burton, 1980,) and oestradiol concentrations are elevated between Day 12 and Day 17 in the pregnant pig (Moeljono et al., 1977), which perhaps indicates

production of oestrogen by the blastocyst. Bazer and Thatcher (1977) proposed that oestrogens produced by the pig blastocyst reduce $PGF_{2\alpha}$ output into the uterine venous blood in pregnant sows, not by inhibiting synthesis of $PGF_{2\alpha}$ but by redirecting its secretion into the uterine lumen. A similar change in the orientation of secretion in the pig endometrium was observed for porcine purple acid phosphatase (Chen, Bazer, Gebhardt and Roberts, 1975). The $PGF_{2\alpha}$ concentration of uterine flushings from non-pregnant pigs was significantly increased by treatment with oestradiol valerate from Days 11-15 (Frank, Bazer, Thatcher and Wilcox, 1978; Geisert, Thatcher, Roberts and Bazer, 1982), which gives support to the theory of oestrogen causing sequestration of $PGF_{2\alpha}$ into the uterine lumen during pregnancy. Oestrogen and PGF concentrations were higher in uterine flushings from pregnant as compared to non-pregnant pigs between Day 12 and Day 18 (Zavy, Bazer, Thatcher and Wilcox, 1980).

Using bilateral perifusion devices to measure prostaglandin secretion by luminal and myometrial surfaces of porcine endometrium, it was found that secretion rates of PGF and PGE_2 were higher from the luminal side for Day-12 pregnant, Day-14 pregnant and Day-14 non-pregnant pigs whereas secretion was higher from the myometrial side for Day-10 pregnant and Day-14 cyclic pigs. Also, prostaglandin secretion from the luminal side of porcine endometrium was increased by oestrogen treatment independent of reproductive status (Gross, Lacroix, Bazer, Thatcher and Harney, 1988a). In addition, porcine conceptuses significantly metabolise $PGF_{2\alpha}$ and PGE_2 (Maule Walker, Patek, Leaf and Watson, 1977), which would protect them from any build up in prostaglandin levels. However, Hunter and Poyser (1982) suggested that a luteotrophic factor might also be involved

in the maintenance of pig corpora lutea during pregnancy as they found significant amounts of $PGF_{2\alpha}$ in the uterine blood from Day-15 and Day-16 pregnant pigs while peripheral progesterone concentrations were not reduced. Immunological tests revealed that the Day 13-16 pig conceptus does not produce any human chorionic gonadotrophin (hCG)-like activity (Powell-Jones, Lester, Polge and Stockell Hartree, 1984).

A luteotrophic factor may also decrease the sensitivity of corpora lutea to $PGF_{2\alpha}$ in the pregnant guinea-pig as, in the 25% of oestradiol treated guinea-pigs which exhibited raised $PGF_{2\alpha}$ levels, plasma progesterone levels were higher than in Day-15 non-pregnant guinea-pigs and no abortions had occurred (Poyser, 1984a). An hCG-like substance was found in guinea-pig placenta from Day 10 of pregnancy with highest mean concentration on Day 18 (Humphreys, Hobson and Wide, 1982), and as hCG prevents the luteolytic effects of $PGF_{2\alpha}$ in hysterectomised guinea-pigs, both morphologically and in terms of progesterone secretion (Tam, Beveridge and Tso, 1982), this hCG-like factor could be responsible for the luteotrophic effect in pregnant guinea-pigs. The lack of increase in the concentration of $PGF_{2\alpha}$ in the uterine venous plasma of the pregnant guinea-pig is not due to redirection of $PGF_{2\alpha}$ from the venous drainage into the uterine lumen, as proposed for the pig (Bazer and Thatcher, 1977), but due to lack of increased endometrial $PGF_{2\alpha}$ synthesis and release. The $PGF_{2\alpha}$ output from guinea-pig uterus superfused in vitro was much lower on Day 15 of pregnancy than on Day 15 of the cycle (Poyser, 1984a) and uterine homogenates from Day-15 bilaterally pregnant guinea-pigs produced significantly less $PGF_{2\alpha}$ than homogenates of Day-15 non-pregnant uterus (Maule Walker and Poyser, 1973, 1974).

Response to oxytocin in pregnant animals

In pregnant sheep and cows, the increase in endometrial oxytocin binding, which normally occurs prior to luteolysis, is not found (McCracken, Glew, Underwood, Gavagan and Roberts, 1979; Jenner et al., 1991). The frequency of oxytocin pulses and $PGF_{2\alpha}$ pulses in the utero-ovarian vein were significantly lower in ewes with persistent corpora lutea during the period of expected luteolysis, compared with ewes which underwent normal luteal regression (Hooper and Thorburn, 1987). However, the amplitude of both oxytocin and $PGF_{2\alpha}$ pulses were similar in both groups of animals, supporting the theory that it is the frequency of $PGF_{2\alpha}$ pulses and not the amplitude which is important in causing luteal regression. The frequent pulses of oxytocin in peripheral plasma of the goat found during the decline in oxytocin concentrations from Day-12 in the non-pregnant animal are absent during the corresponding period of pregnancy (Homeida and Cooke, 1983). The $PGF_{2\alpha}$-releasing response to oxytocin in the cyclic mare and heifer was abolished in pregnant animals (Goff and Pontbriand, 1985; Lafrance and Goff, 1985a, 1985b).

In addition, oxytocin increased the concentration of IP_3 and total IP in endometrium from Day-16 cyclic but not from Day-16 pregnant ewes (Mirando et al., 1990a) and greater incorporation of [^{3}H]-inositol into IP_3 and total IP in response to oxytocin was detected for cyclic than for pregnant ewes (Mirando, Ott, Harney and Bazer, 1990b). Therefore, the prevention of luteal regression in pregnant sheep may in part be due to suppression of the pulsatile release of oxytocin.

Maternal recognition of pregnancy

In the domestic ruminants, implantation of the conceptus occurs fairly late, at a time when corpus luteum regression has already occurred in the cyclic animal, and the embryo must therefore save the corpus luteum while still floating free in the uterine lumen. In rodents and primates, including the human, implantation occurs before the time at which the corpus luteum normally regresses. The mechanism by which the conceptus "rescues" the corpus luteum may therefore vary between early and late implanting species.

Conceptuses obtained from guinea-pigs on Days 6, 9 or 10 of pregnancy when grafted onto the spleen of non-pregnant guinea-pigs did not delay the recurrence of oestrus, but Day-11 or Day-12 conceptuses did extend the length of the oestrous cycle in recipients (Bland and Donovan, 1969b). Therefore, the guinea-pig conceptus secretes a systemically acting substance which prevents luteal regression from Day 11.

Transfer of sheep embryos to non-pregnant ewes also saved the corpus luteum if carried out before the recipient reached Day 13 of the cycle, but transferral on or after this time resulted in failure of pregnancy as the corpus luteum had already begun to regress (Moor and Rowson, 1966b). Removal of sheep embryos before Day 12 of pregnancy led to oestrus occurring at the normal time whereas a delay in removal until Day 13, 14 or 15 resulted in a marked extension of luteal function (Moor and Rowson, 1966c). Therefore, the sheep conceptus produces a "corpus luteum-saving factor" from Day 12. Day-12 and Day-13 non-pregnant ewes with Day-12 or Day-13 embryos transferred to the uterine horn ipsilateral to the corpus luteum exhibited prolonged cycles, no decline in peripheral plasma progesterone concentrations and no pulsatile peaks of $PGF_{2\alpha}$

(Peterson et al., 1976).

In the cow, pregnancies were obtained in recipients of embryos up to Day 16 of the cycle, but not in those cows receiving embryos on Day 17 (Betteridge, Eaglesome, Randall and Mitchell, 1980) when the corpus luteum had already begun to regress. Removal of conceptuses on Day 17 and after, but not on Day 13 or Day 15, resulted in a significant prolongation of interoestrous intervals in the goat (Gnatek, Smith, Duby and Godkin, 1989). Therefore, the goat conceptus produces an antiluteolytic factor from Day 16. Removal of the equine conceptus on Day 16 or Day 24 resulted in an extended interoestrous interval, but the lifespan of the corpus luteum was not significantly extended in animals from which conceptuses were removed by uterine lavage on Day 10, 12 or 14 (Kooistra and Ginther, 1976; Hershman and Douglas, 1979). Removal of conceptuses from pregnant mice on Day 10 or later gave interoestrous intervals which were approximately as long as gestation and longer that those seen when conceptuses were removed on Day 9 or earlier (Critser et al., 1980).

Infusion of homogenised, Day 11-14 conceptuses into the uterus of non-pregnant guinea-pigs from Day 8 elongated the cycle to 20 days but had no effect on luteal life span as judged by plasma progesterone levels which fell as normal on Days 12-13 (Maule Walker and Poyser, 1976). Intrauterine infusion of homogenates from Day-14 or Day-15 sheep embryos increased cycle lengths whereas infusion of homogenates from Day-25 sheep embryos or Day-14 pig embryos did not (Rowson and Moor, 1967).

Injection into the uterus of homogenates or extracts from 14-16 day old trophoblasts maintained the corpus luteum in cyclic recipient ewes, but homogenates from embryos at 21-23 days of

pregnancy did not (Martal, Lacroix, Loudes, Saunier and Wintenberger-Torres, 1979). The sheep conceptus, therefore, secretes an antiluteolytic factor from Day 13 which is not present at Day 21. Embryo transferrence to intact ewes resulted in maintenance of the corpus luteum whether they were transferred to the ipsilateral horn or the contralateral horn, but embryos transferred to sheep with one surgically isolated uterine horn maintained luteal function only if they were placed in the ipsilateral horn (Moor and Rowson, 1966d). When embryos were transferred to one isolated horn of recipient ewes that had corpora lutea in both ovaries only the corpora lutea adjacent to the gravid horn were maintained. Thus, the sheep conceptus does not produce a systemically acting antiluteolytic substance but overcomes the luteolytic effect of the uterus in a local manner.

In bilaterally ovulating, unilaterally pregnant ewes, with the uterine horns separated and ligated to produce one gravid and one non-gravid horn and the main uterine vein from one side then anastomosed to the corresponding vein on the other side (i.e. gravid to non-gravid or non-gravid to gravid), the corpus luteum regressed when the ipsilateral vein contained blood from only the non-gravid horn but was maintained when the ipsilateral vein contained venous blood from the gravid horn (Mapletoft, Del Campo and Ginther, 1975). Thus, the sheep conceptus secretes a substance into the uterine lumen which signals its presence to the mother and prevents luteal regression, but this substance does not act systemically on the non-pregnant horn of unilateral pregnancy.

Injection of plasma from Day-10, but not from Day-8 or Day-12, pregnant mice and extracts of conceptuses from Day 10 and 11, but not from Day 8 or 9, of pregnancy inhibited the occurrence of

vaginal oestrus, stimulated mammary development and elevated plasma progesterone levels in non-pregnant mice (Critser et al., 1980). Therefore by Day 10 of pregnancy, the mouse conceptus produces substances which cause extension of luteal life span. It appears that in many species, the embryo or conceptus secretes substances which prevent luteal regression by acting on the uterus and/or ovary. However, the infusion of porcine embryonic membranes into the uterus of non-pregnant pigs did not extend the life span of the corpora lutea (Longnecker and Day, 1972).

Corpus luteum maintenance during unilateral pregnancy

Unilateral pregnancy resulted in bilateral luteal maintenance in guinea-pigs (Bland and Donovan, 1967; Deanesly, 1967), although Oxenreider and Day (1967) found the corpora lutea adjacent to the non-gravid horn to be smaller than the corpora lutea in the ovary adjacent to the gravid horn. Also, in unilaterally pregnant guinea-pigs with the non-pregnant horn isolated at the cervical end (i.e. there is no connection between the two horns), endometrial $PGF_{2\alpha}$ synthesis is reduced in the non-pregnant horn as well as the pregnant horn and the corpora lutea are maintained in both ovaries (Poyser and Maule Walker, 1979). These results indicate that the guinea-pig conceptus produces a systemically acting antiluteolytic factor. In bilaterally ovulated, unilaterally pregnant sheep with the non-pregnant horn isolated from the pregnant horn, corpora lutea in the ovary adjacent to the non-pregnant horn regress (Moor, 1968), indicating that $PGF_{2\alpha}$ synthesis in the non-pregnant horn has not been inhibited. This suggests that the antiluteolytic factor from the sheep embryo does not circulate or act systemically.

Unilateral pregnancy, whether due to congenital occlusion of one

horn (Nablandov, 1952) or surgery prior to breeding (Rathmacher and Anderson, 1963), cannot be established from conception nor be continued in the pig. Pregnancy fails in bilaterally pregnant pigs in which the embryos are removed from one horn and the horn ligated on Day 4 or 10, whereas this treatment carried out between Day 12 and 50 of pregnancy has no effect (Dhinsda and Dziuk, 1968). Pigs made unilaterally pregnant with fertilised ova on Day 2 showed unilateral corpus luteum maintenance in the ipsilateral horn, whereas pigs made unilaterally pregnant on Day 12 exhibited bilateral maintenance of the corpora lutea (Niswender, Dziuk, Kaltenbach and Norton, 1970). Therefore, the antiluteolytic factor from pig conceptuses does not act systemically and, as the uterine luteolytic factor in pigs apparently acts systemically (Anderson, Butcher and Melampy, 1961) it seems that, during unilateral pregnancy, the release of $PGF_{2\alpha}$ by the non-pregnant horn is capable of causing corpus luteum regression in the ovary ipsilateral to the pregnant horn and terminating pregnancy. Therefore, embryos need to be present in both horns up to Day 12 for bilateral luteal maintenance and continuation of pregnancy in the pig. Indeed, infusion of pig embryonic membranes overcame the detrimental effects of unilateral pregnancy when the infused material had access to the entire sterile horn and resulted in corpora lutea which were maintained bilaterally (Longnecker and Day, 1972).

Pregnancy failure occurs in mares in which conceptus mobility is artificially restricted by ligating one horn (i.e. unilateral pregnancy) unless progestational support is given (McDowell, Sharp, Peck and Cheves, 1985), and this suggests that the antiluteolytic factor from the equine conceptus does not act systemically. Although the horse normally gives birth to only one foal, intrauterine

migration of the horse conceptus is extensive (Ginther, 1983) and this presumably allows it's secretions to affect the entire uterine endometrium in order to reduce $PGF_{2\alpha}$ secretion and allow establishment of pregnancy.

It appears therefore that the antiluteolytic factor secreted by the sheep, pig and horse conceptus cannot act systemically to reduce $PGF_{2\alpha}$ synthesis in a non-pregnant horn and, presumably, does not enter the maternal vasculature. However the antiluteolytic factor from the guinea-pig conceptus does act systemically and therefore may act by a different mechanism from that in the sheep, pig and horse.

Conceptus protein secretion

The antiluteolytic factor secreted by Day 14-16 sheep embryonic homogenates is thermolabile and inactivated by pronase (Martal et al., 1979), implying that it is a protein. This led to investigation of protein secretion by conceptuses at the time at which luteal maintenance occurs. The major protein product secreted by Day-13 sheep blastocysts in culture consisted of three closely similar isoelectric species each with molecular weight (Mr) of about 17,000 Da and a pI of approximately 5.5. These proteins predominated until Day 23, after which time they could not be detected (Godkin, Bazer, Moffat, Sessions and Roberts, 1982a). Culture medium from cow conceptuses on Day 16, 19, 22 and 24 of gestation was enriched in low molecular weight, acidic polypeptides which were not prominent products of Day 29 or Day 69 tissues (Bartol, Roberts, Bazer, Lewis, Godkin and Thatcher, 1985). Day-10.5 and Day-12 cultured pig blastocysts released a group of low molecular weight acidic polypeptides (Mr 20,000-25,000 Da, pI 5.6-6.2), although between Day

13 and Day 16 the major proteins detected were basic and in the Mr range 35,000-50,000 Da (Godkin, Bazer, Lewis, Geisert and Roberts, 1982b). Day 13 pig conceptuses released two major radiolabelled proteins of Mr 23,000 Da and 26,000 Da and Day 14-16 conceptuses released these proteins as well as proteins of Mr 14,000, 19,000, 44,000, 50,000 and 88,000 (Powell-Jones et al., 1984). The predominant basic protein synthesised and secreted by Day 14-17 pig conceptuses in culture was a glycoprotein of Mr 43,100 Da or 42,800 Da under denaturing or native conditions, respectively (Baumbach, Climer, Bartley, Kattesh and Godkin, 1988). The major protein synthesised by goat conceptus up to Day 21 had two isotypes of molecular weight 17,000 Da and pI 5.2-5.7 (Gnatek et al., 1989). Therefore, the sheep, cow, pig and goat conceptus all secrete low molecular weight acidic proteins at the time of maternal recognition of pregnancy which are not found at later stages of gestation.

Day-12 and Day-14 horse conceptuses in culture secreted an array of five proteins that ranged in Mr from around 50,000 Da to more than 400,000 Da, but by Day 16 the pattern of protein secretion had changed markedly to include several serum-like proteins such as transferrin and α-foetoprotein (Fazleabas and McDowell, 1983).

Identification of trophoblastic proteins

Cyclic ewes which had a concentrated solution of "total sheep conceptus culture medium protein" infused into the uterine lumen between Day 12 and Day 18 did not ovulate and their peripheral progesterone levels remained elevated (Godkin, Bazer, Thatcher and Roberts, 1984a). The major protein present in the culture medium was purified and named ovine trophoblast protein-1 (oTP-1; Godkin, Bazer and Roberts, 1984b). oTP-1 was the major conceptus mRNA between Day

13 and Day 21 in sheep, a time corresponding to that at which the conceptus acts to extend luteal life-span (Hansen, Anthony, Bazer, Baumbach and Roberts, 1985). Receptors for oTP-1 were detected in membrane preparations from sheep endometrial homogenates (Godkin et al., 1984b). Using immunocytochemical techniques, oTP-1 was shown to be localised in the upper glandular and superficial epithelium of the endometrium but not in deep glands, stroma or myometrium, and [^{125}I]-labelled oTP-1 infused into the uterus of Day-12 non-pregnant ewes revealed that the majority of the radioactivity was retained in the uterus with only very small amounts of protein entering the maternal vasculature (Godkin et al., 1984b). In addition, there was no significant association of [^{125}I]-labeled oTP-1 with the corpora lutea, ovaries or other tissues and oTP-1 did not stimulate progesterone production by dispersed luteal cells from Day-12 cycling ewes (Godkin et al., 1984b). oTP-1 selectively stimulated the synthesis of six polypeptides from endometrial explants of non-pregnant ewes (Godkin et al., 1984b). These findings suggest that oTP-1 binds to and acts on the sheep endometrium in an antiluteolytic manner rather than having a direct luteotrophic effect on the corpus luteum.

Intrauterine injections of pooled, bovine conceptus secretory proteins (bCSP) on Days 15.5-21 extended corpus luteal life-span and interoestrous interval in the non-pregnant cow, and reduced $PGF_{2\alpha}$ levels in the vena cava (Knickerbocker, Thatcher, Bazer, Drost, Barron, Fincher and Roberts, 1986a). Transfer of trophoblastic vesicles from Day 11-13 sheep conceptuses to Day-12 recipient cows resulted in extension of luteal lifespan in a significant number of animals, and reciprocal interspecies transfer of bovine trophoblastic tissue to recipient ewes had a similar effect (Heyman,

Camous, Fevre, Meziou and Martal, 1984). These results suggested that the trophoblasts of sheep and cows produced a functionally similar antiluteolytic substance. Indeed, using a rabbit antiserum to oTP-1, a group of 6-8 polypeptides from culture medium of bovine conceptuses was specifically immunoprecipitated and the immunoprecipitated polypeptides constituted the major secretory products of Day 16-25 bovine conceptuses (Helmer, Hansen, Anthony, Thatcher, Bazer and Roberts, 1987). The bovine trophoblastic protein-1 (bTP-1) complex comprised of 3 major and 2 or more minor isoelectric species (pI 5.8-6.8) (Godkin, Lifsey and Gillespie, 1988a). Purified bovine trophoblast protein-1 (bTP-1) obtained from Day 17-18 cow conceptuses in culture infused intrauterinely into non-pregnant cows from Day 15.5-21 lengthened the oestrous cycle, maintained elevated progesterone levels and reduced $PGF_{2\alpha}$ concentrations in the vena cava (Helmer, Hansen, Thatcher, Johnson and Bazer, 1989a). [^{125}I]-bTP-1 was shown to bind specifically to bovine uterine epithelial cells in culture (Godkin, Lifsey, Fujii and Baumbach, 1988b).

Intrauterine infusion of conceptus secretory proteins (CSP) from Day-15 pregnant pigs into cyclic pigs between Day 12 and Day 15 did not lengthen interoestrous intervals compared to serum protein (SP)-treated pigs (Harney and Bazer, 1989), and the dominant 22,000-24,000 Mr cluster of acidic proteins secreted by porcine conceptuses on Day 11 did not cross-react with antiserum against oTP-1 (Cross and Roberts, 1989). There were no proteins of similar isoelectric point or molecular weight range to oTP-1 in conceptus membranes from the horse prior to Day 15, and there was no immunological cross-reactivity of conceptus-conditioned media from equine conceptus cultures in an oTP-1 radioimmunoassay (Sharp,

McDowell, Weithenauer, Franklin, Mirando and Bazer, 1989). However, three low molecular weight acidic proteins secreted by Day-17 goat conceptus cultures were immunoprecipitated by anti-oTP-1 serum (Gnatek et al., 1989).

Uterine flushings from pregnant ewes contained oTP-1 between Day 14 and Day 24 of pregnancy, but not on Day 12, and all cyclic ewes tested negatively for oTP-1 (Kazemi, Malathy, Keisler and Roberts, 1988). In pregnant sheep in which the conceptus had been confined to one horn, oTP-1 was not detected in flushings from the non-gravid horn (Kazemi et al., 1988) indicating that oTP-1 does not act systemically and is confined to the region of the conceptus. bTP-1 was found in the uterine flushings of pregnant cows using antiserum to oTP-1 (Kazemi et al., 1988).

Sheep, cow and goat conceptuses produce trophoblastic proteins which are antiluteolytic and are immunologically similar. The pig, however, does not secrete "corpus luteum lifespan extending proteins" from its conceptus, nor were the proteins which were secreted by the pig or the horse conceptus similar to oTP-1 .

Homology of oTP-1 and bTP-1 with α-interferon

Sequencing of the N-terminal amino acids of oTP-1 revealed many similar or homologous amino acids to human α-interferon (Stewart, McCann, Baker, Lee, Lamming and Flint, 1987) and bovine α-interferon II (Charpigny, Reinaud, Huet, Gullimot, Charlier, Pernollet and Martal, 1988).

The primary amino acid sequence of oTP-1 inferred from cloned complementary DNA (cDNA) exhibited high sequence homology with α-interferons from the human, cow, mouse, rat and pig, but the most extensive sequence homology was with bovine α-interferon (70.3%)

(Imakawa, Anthony, Kazemi, Marotti, Polites, and Roberts, 1987). No ovine interferon has ever been isolated, nor has any ovine interferon gene been cloned and sequenced. The inferred amino-acid sequence of bTP-1 from a cDNA library shared 80% identity with oTP-1, between 45-55% with human, rodent, porcine and bovine interferons of the α-1 subfamily and about 69% identity to bovine α-interferon II (Imakawa, Hansen, Anthony, Polites, Marotti and Roberts, 1988; Imakawa, Hansen, Malathy, Anthony, Polites, Marotti and Roberts, 1989).

[^{125}I]-labeled human α-interferon was inhibited from binding to high affinity sites in membrane fractions of sheep endometrium just as effectively by oTP-1 as by unlabelled human α-interferon (Stewart et al., 1987), and [^{125}I]-bovine recombinant α-interferon bound one fifth as avidly to ovine endometrium as [^{125}I]-human α-interferon (Flint, Lamming, Stewart and McCann, 1988). Bovine recombinant α-interferon infused into the uterine horn ipsilateral to the corpus luteum in ewes from Day 9 to Day 19 extended luteal phase lengths and lowered mean PGFM concentrations (Lamming, Parkinson and Flint, 1988). The infusion of recombinant bovine α-interferon into the uterus of cyclic cows from Day 15.5 to Day 21 after oestrus delayed luteolysis from 22.8 days to 26.8 days (Plante, Hansen and Thatcher, 1988), and measurement of circulating progesterone levels confirmed that this was due to an increase in corpus luteum lifespan.

Both human leucocyte-derived and fibroblast-derived interferon are potent inhibitors of human peripheral blood mononuclear cell prostaglandin synthesis at low physiological concentrations (Dore-Duffy, Perry and Kuo, 1983). Similarly, human α-interferon II and purified oTP-1 significantly attenuated $PGF_{2\alpha}$ and PGE release

from cultured ovine endometrial cells and stimulated the synthesis and secretion of the same "pregnancy related" proteins (Salamonsen, Stuchbery, O'Grady, Godkin and Findlay, 1988). However, interferon was approximately 100 times more potent than oTP-1 in inhibiting the release of $PGF_{2\alpha}$ and PGE_2 from cultured ovine endometrial cells (Salamonsen, Manikhot, Healy and Findlay, 1989). Thus, α-interferons are not only immunologically alike to oTP-1 and bTP-1 but can also bind to endometrial oTP-1 and bTP-1 receptors, and cause functionally similar events such as extension of the luteal cycle and inhibition of uterine $PGF_{2\alpha}$ production.

The 22,000-24,000 Da Mr cluster of acidic proteins from Day-11 porcine conceptuses cross-reacted on immunoblots with antiserum against human α-interferon but not against oTP-1 (Cross and Roberts, 1989). Interferon-like antiviral activity was present in porcine conceptus culture medium and uterine flushings from Day 11-17 of pregnancy but not in flushings prior to Day 11 of pregnancy or in flushings from Day-12 non-pregnant animals, and the antiviral activity co-eluted with the 22,000-24,000 Da Mr protein (Cross and Roberts, 1989). Therefore, the pig conceptus produces an interferon-like protein but it is not immunologically related to oTP-1. Bioassay of equine uterine flushings as well as conceptus conditioned media from equine conceptus cultures did not reveal any antiviral activity (Sharp et al., 1989). Antiviral activity indicative of interferon was not found in medium containing proteins from Day-5 mouse blastocysts or trophoblast outgrowths (Baker and Neider, 1990). Therefore, interferons are probably not involved in the maternal recognition of pregnancy at the time of implantation in the mouse.

These results suggest that the maternal recognition of pregnancy

in the cow and sheep is due to the release from the trophoblast of an interferon-like protein which lengthens the lifespan of the corpus luteum by preventing the synthesis of endometrial $PGF_{2\alpha}$. Caprine trophoblast protein-1 (cTP-1) produced by the goat conceptus is presumably an interferon-like protein as well since it is immunologically similar to oTP-1 (Gnatek et al., 1989). Whether this mechanism is specific to domestic ruminants or present in other species is unknown. However, antiviral activity indicative of interferon has not been found in the mouse trophoblast or the horse conceptus.

Mechanism of action of ovine conceptus secretory proteins (oCSP) and oTP-1

As pulsatile $PGF_{2\alpha}$ secretion from the uterus at luteolysis in the sheep is thought to be brought about by the binding of ovarian oxytocin to uterine receptors, it was suggested that oTP-1 might exert its antiluteolytic effect by an action on the oxytocin receptor. Indeed, uterine production of $PGF_{2\alpha}$ in response to oestradiol and oxytocin was suppressed in pregnant ewes, and in ewes treated on Days 12-14 with oCSP (Fincher, Bazer, Hansen, Thatcher and Roberts, 1986) or purified oTP-1 (Vallet, Bazer and Fliss, 1987a). Ewes treated with oCSP or oTP-1 on Days 12, 13 and 14 of the cycle had longer interoestrous intervals compared to ewes treated with serum proteins (SP) or with oCSP from which the oTP-1 had been removed, and the stimulation of uterine $PGF_{2\alpha}$ production in response to oestradiol and oxytocin was reduced (Vallet, Bazer, Fliss and Thatcher, 1988). It was proposed that oTP-1 might inhibit oxytocin receptor synthesis and, therefore, prevent pulsatile secretion of $PGF_{2\alpha}$.

Oxytocin stimulated IP_3 and total IP turnover in the endometrium of SP-treated but not oCSP-treated Day-16 cyclic ewes (Mirando et al., 1990a). SP-infused and oCSP-infused cyclic ewes had similar peripheral plasma PGFM profiles in response to oxytocin on Day 11, but SP-treated ewes became more responsive to oxytocin between Days 13 and 15 than oCSP-treated ewes (Mirando et al., 1990b). SP-treated ewes also had greater endometrial incorporation of [^{3}H]-inositol into IP_3 and total IP in response to oxytocin than did oCSP-treated ewes on Day 16. Therefore, oTP-1 prevents the development of endometrial responsiveness to oxytocin perhaps by an inhibitory action on the oxytocin receptor.

Turnover of PI in the endometrium of Day-15 cyclic ewes was unaffected by treatment with oTP-1 alone (Vallet and Bazer, 1989). Co-treatment of endometrium from Day-15 cyclic ewes with oTP-1 and oxytocin inhibited the stimulatory effects of oxytocin on PI turnover, while treatment with oTP-1 before and during oxytocin administration had no effect. oTP-1 had no effect alone or in combination with oxytocin on oxytocin-stimulated PI turnover in the endometrium of ovariectomised ewes which had been progesterone for 10 days and then oestrogen for 2 days. In ovariectomised ewes treated with progesterone, oestrogen, oestrogen plus progesterone or neither steroid for 10 days, oTP-1 alone had no effect on endometrial PI turnover, while co-treatment of endometrium with oxytocin and oTP-1 stimulated PI turnover in ewes treated with progesterone, but not with progesterone and oestrogen. Pretreatment of endometrium with oTP-1 stimulated PI turnover when ewes were treated with progesterone or progesterone plus oestrogen. Pretreatment of endometrium with oxytocin and then treatment with oTP-1 inhibited PI turnover compared to treatment with oxytocin

alone. Intrauterine treatment of ovariectomised steroid treated ewes with oTP-1 on Days 10-12 of steroid treatment had no effect on PI turnover in endometrium on Day 13, and oxytocin could stimulate PI turnover in the endometrium of these ewes. These results indicated that the antiluteolytic effects of oTP-1 are not mediated by inhibiting effects of oxytocin on PI turnover if oxytocin receptors are present.

However, Ott, Fliss and Davis (1989) found that intra-uterine infusion of oCSP into ovariectomised, progesterone-treated ewes on Day 11, 12, 13 and 14 of treatment abolished oxytocin-stimulated PGFM output in peripheral plasma and inhibited endometrial IP turnover only if progesterone treatment was maintained to Day 15 and not if it was stopped at Day 10. These results indicate that inhibition of endometrial response to oxytocin by oCSP required the presence of progesterone and may explain the lack of an effect of oTP-1 in ovariectomised ewes only treated for 10 days with progesterone seen by Vallet and Bazer (1989). Further evidence for the luteolytic effect of oTP-1 being mediated by an effect on the oxytocin receptor was demonstrated when oCSP or bovine recombinant interferon treatment of sheep on Days 12, 13 and 14 of the cycle suppressed both endometrial oxytocin receptor concentrations and oxytocin-induced increase in peripheral plasma PGFM concentrations compared to SP-treatment (Vallet, Lamming and Batten, 1990b). oCSP treatment also suppressed oxytocin receptor concentrations in ovariectomised progesterone plus oestrogen treated ewes when given on Days 10, 11 and 12 of progesterone treatment compared to SP or bovine recombinant interferon treatment. Oxytocin stimulated greater release of PGF from oTP-1-treated than bovine serum albumin (BSA)-treated endometrium when using an in vitro perifusion system

(Vallet, Gross, Fliss and Bazer, 1989). However, when cyclic ewes were treated in vivo with oTP-1 on Days 12, 13 and 14 and endometrium was collected on Day 15, the endometrial secretion of PGF in response to oxytocin was attenuated. Thus, the physiologically relevant treatment of long-term exposure of the endometrium to oTP-1 will inhibit the release of luteolytic $PGF_{2\alpha}$ in response to oxytocin in the sheep.

Effect of conceptus secretory proteins (CSP) on endometrial protein synthesis

Both bovine and ovine conceptus secretory proteins have an effect on endometrial protein synthesis. Godkin et al. (1984b) reported that oTP-1 increased the synthesis of at least six polypeptides in endometrial explants from Day-12 cyclic ewes. Further work demonstrated that oTP-1 enhanced the secretion of eleven proteins and reduced the secretion of six proteins in endometrium from Day-12 cyclic ewes (Vallet, Bazer and Roberts, 1987b). Most of the proteins increased by oTP-1 were acidic and in particular a protein with Mr of about 70,000 Da and pI of about 4 was increased 400%. Endometrial cells from ovariectomised, oestrogen and progesterone-treated ewes had the synthesis of five proteins stimulated by treatment with oTP-1 or human interferon α-2 in vitro (Salamonsen et al., 1988). Four of these proteins are also present following culture of epithelial endometrial cells from ewes on Day 13 of pregnancy, or following in vitro culture of cells from unmated ewes on Day 13 of the oestrous cycle with added concentrated medium from culture of Day 15 sheep blastocysts (Salamonsen, Doughton and Findlay, 1986). The functions of endometrial proteins induced by oTP-1 are as yet unknown, but it is interesting to speculate that they may serve a

role in the antiluteolytic mechanism either by an action on the oxytocin receptor or by directly inhibiting endometrial $PGF_{2\alpha}$ synthesis.

The latter mechanism may be responsible for the antiluteolytic action of bTP-1 in the cow. In cattle, intracellular inhibitors of prostaglandin synthesis have been reported in uterine and placental tissues (Wlodawer, Kindahl and Hamberg, 1976; Shemesh, Hansel and Strauss, 1984). Experiments were devised to determine if endometrial tissues from Day 17 of pregnancy in cattle contained an endogenous intracellular inhibitor of prostaglandin synthesis that was absent or reduced at Day 17 of the oestrous cycle. Indeed, endometrial intracellular preparations from Day-17 pregnant cows markedly decreased PGF synthesis by cotyledonary microsomes from parturient cows (this preparation was utilized as a prostaglandin-generating system) compared to intracellular preparations from Day-17 nonpregnant cows (Gross, Thatcher, Hansen, Johnson and Helmer, 1988b). The inhibitor was proteinaceous and it was proposed that it might be induced by secretory proteins from the conceptus. Treatment with bovine conceptus secretory proteins (bCSP) reduced incorporation of $[^3H]$-leucine into proteins secreted by endometrial explants from Day-17 cyclic cows but selectively induced the secretion of two proteins of 13 and 10 kDa (Gross, Plante, Thatcher, Hansen, Helmer and Putney, 1988c). Secretion of PGF, but not of PGE_2, was decreased in endometrium from Day-17 cyclic cows treated with bCSP. In addition, synthesis of PGF by the cotyledonary PG generating system was decreased when incubated with the cytosol from endometrium treated with bCSP, but was unaltered by cytosol from untreated tissues. Therefore products produced by the bovine conceptus are capable of regulating endometrial protein and

prostaglandin synthesis in a way which could prevent luteolysis in vivo.

To see whether the effects of bCSP were mediated by bTP-1, the effects of each were compared on the PG and protein secretion of endometrial explants from Day-17 cyclic cows. Both bCSP and bTP-1 decreased the release of radiolabelled proteins into the medium and the incorporation of radiolabelled proteins into the tissue (Helmer, Gross, Newton, Hansen and Thatcher, 1989b). Both bCSP and bTP-1 decreased PGF secretion by endometrial explants and induced the synthesis of an intracellular inhibitor of PGF synthesis from cotyledonary microsomes. From these results, it appears that bTP-1 is the active component of bCSP with respect to regulation of prostaglandin secretion. However, the secretion of a protein of Mr 14,900 Da by the endometrium was enhanced by bCSP treatment but not by bTP-1 treatment (Helmer et al., 1989b); suggesting that the increase in secretion of this protein is caused by a component of bCSP distinct from bTP-1.

Summary and aims in the pregnant guinea-pig

The secretion of progesterone from the corpus luteum in the guinea-pig is necessary for the maintenance of pregnancy for the first four weeks (Heap and Deanesly, 1966; Csapo et al., 1981). Therefore, during pregnancy, the lifespan of the corpus luteum must be extended beyond its normal period of approximately 17 days. Indeed the secretion of luteolytic $PGF_{2\alpha}$ remains low on Day 15 of pregnancy compared to Day 15 of the cycle (Blatchley et al., 1975a, 1975b; Antonini et al., 1976). This is not due to redirection of $PGF_{2\alpha}$ secretion towards the uterine lumen as proposed in the pig (Bazer and Thatcher, 1977), since $PGF_{2\alpha}$ release from the

guinea-pig uterus superfused *in vitro* is much lower on Day 15 of pregnancy than on Day 15 of the cycle (Poyser, 1984a). As Day-11 or 12, but not Day-6, 9 or 10 guinea-pig conceptuses extend luteal cycles when grafted onto the spleens of normal cyclic guinea-pigs (Bland and Donovan, 1969b), it would appear that the guinea-pig conceptus produces some antiluteolytic substance from Day 11.

This thesis aims to determine the nature of the antiluteolytic mechanism in pregnant guinea-pigs and in particular to compare it with the mechanism of maternal recognition of pregnancy in domestic ruminants. In these species luteal maintenance is caused by the release of an interferon-like protein from the trophoblast which decreases the synthesis of $PGF_{2\alpha}$ by the endometrium. Consequently, the studies in this thesis have investigated whether the guinea-pig conceptus also produces a protein which acts like oTP-1 and therefore may be related to α-interferon.

SECTION 2

MATERIALS AND GENERAL METHODS

Introduction

Many of the procedures carried out in this thesis are common to several experiments and, so the details of these procedures are given in this section.

2:1 Materials used

Chemicals	Source
Acrylamide	B.D.H. Chemicals Ltd, U.K.
Ammonium persulphate	Sigma Chemical Co., Poole, Dorset, U.K.
Amphoterecin B (Fungizone^{TM})	Flow Laboratories, Irvine, U.K.
Arachidonic acid	Sigma Chemical Co., Poole, Dorset, U.K.
Bis-acrylamide (N,N'-methylene-bis-acrylamide)	B.D.H. Chemicals Ltd, U.K.
Bovine serum albumin (BSA)	Sigma Chemical Co., Poole, Dorset, U.K.
Bromophenol Blue	B.D.H. Chemicals Ltd, U.K.
Calcium chloride	B.D.H. Chemicals Ltd, U.K.
Cholera toxin	Sigma Chemical Co., Poole, Dorset, U.K.
Coomassie Blue R	Sigma Chemical Co., Poole, Dorset, U.K.
D-Glucose	B.D.H. Chemicals Ltd, U.K.
Disodium ethylene diamine tetra-acetic acid (EDTA)	B.D.H. Chemicals Ltd, U.K.
Dithiothreitol	Sigma Chemical Co., Poole,

	Dorset, U.K.
Formic acid	B.D.H. Chemicals Ltd, U.K.
Gelatine	B.D.H. Chemicals Ltd, U.K.
Glutamine	Flow Laboratories, Irvine, U.K.
Glycerol	Sigma Chemical Co., Poole, Dorset, U.K.
Glycine	B.D.H. Chemicals Ltd, U.K.
Hydrochloric acid (HCl)	B.D.H. Chemicals Ltd, U.K.
Indomethacin	Merck, Sharpe and Dohme Ltd, Herts., U.K.
Interferon (human-α, lymphoblastoid, $1x10^7$ I.R.U./mg protein)	Sigma Chemical Co., Poole, Dorset U.K.
Kanamycin	Flow Laboratories, Irvine, U.K.
L-leucine	Sigma Chemical Co., Poole, Dorset, U.K.
Magnesium sulphate	B.D.H. Chemicals Ltd, U.K.
Medium 199 (plus Earle's salts)	Flow Laboratories, Irvine, U.K.
Mercaptoethanol	B.D.H. Chemicals Ltd, U.K.
Neomycin sulphate	Sigma Chemical Co., Poole, Dorset, U.K.
Nonidet P-40	LKB Products, Sweden.
Pertussis toxin	Sigma Chemical Co., Poole, Dorset, U.K.
$PGF_{2\alpha}$ and PGE_2	Gift of Dr. J. Pike, Upjohn Co., Kalamazoo, U.S.A.
6-keto-$PGF_{1\alpha}$	Sigma Chemical Co., Poole,

	Dorset, U.K.
Phospholipase A_2 (from Naja naja venom)	Sigma Chemical Co., Poole, Dorset, U.K.
Polyacrylamide	Aldrich Chemical Co. Inc., Milwaukee, U.S.A.
Potassium chloride	B.D.H. Chemicals Ltd, U.K.
Potassium dihydrogen orthophosphate	B.D.H. Chemicals Ltd, U.K.
PPO (2,5-diphenyloxazole)	B.D.H. Chemicals Ltd, U.K.
Silica gel (100-200 mesh)	Koch-Light Laboratories Ltd, U.K.
Sodium acetate	Fison's Scientific Equipment, Leics., U.K.
Sodium azide	Hopkin and Williams Ltd, Essex, U.K.
Sodium chloride	B.D.H. Chemicals Ltd, U.K.
Sodium dihydrogen orthophosphate	B.D.H. Chemicals Ltd, U.K.
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co., Poole, Dorset, U.K.
Sodium fluoride	Fison's Scientific Equipment, Leics., U.K.
Sodium hydrogen carbonate	B.D.H. Chemicals Ltd, U.K.
Sodium hydroxide	B.D.H. Chemicals Ltd, U.K.
Tetramethylethylenediamine (TEMED)	B.D.H. Chemicals Ltd, U.K.
TMB-8 (3,4,5-trimethylbenzoic acid, 8-(dimethylamino)octyl ester hydrochloride)	Sigma Chemical Co., Poole, Dorset, U.K.
Trichloroacetic acid	Fison's Scientific Equipment Leics., U.K.

Trifluoperazine	Sigma Chemical Co., Poole, Dorset, U.K.
Tris (hydroxymethyl)methylamine (Tris)	B.D.H. Chemicals Ltd, U.K.
Urea	B.D.H. Chemicals Ltd, U.K.
W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride)	Sigma Chemical Co., Poole, Dorset, U.K.

Solvents	Source
Acetic acid, glacial	B.D.H. Chemicals Ltd, U.K.
Acetonitrile	Rathburn Chemicals Ltd, U.K.
Chloroform	Rathburn Chemicals Ltd, U.K.
1,4-Dioxan	B.D.H. Chemicals Ltd, U.K.
2-Ethoxyethanol	B.D.H. Chemicals Ltd, U.K.
Ethyl acetate*	B.D.H. Chemicals Ltd, U.K.
Hexane	Rathburn Chemicals Ltd, U.K.
Methanol	B.D.H. Chemicals Ltd, U.K.
Toluene	B.D.H. Chemicals Ltd, U.K.

*redistilled prior to use

Radioactive compounds

[5,6,8,9,11,12,14,15(n)-^{3}H] prostaglandin $F_{2\alpha}$ (specific activity 179Ci/mmol).

[5,6,8,11,12,14,15(n)-^{3}H] prostaglandin E_2 (specific activity 185-200Ci/mmol).

6-keto [5,8,9,11,12,14,15(n)-^{3}H] prostaglandin $F_{1\alpha}$ (specific activity 151-170Ci/mmol).

All [^{3}H] PG stock solutions were diluted to 5μCi/ml and stored at -20°C before using for radioimmunoassay. [^{3}H]-$PGF_{2\alpha}$ and

[^{3}H]-PGE_2 were diluted in methanol and [^{3}H]-6-keto-$PGF_{1\alpha}$ was diluted in acetonitrile:water (9:1).

L-[4,5-^{3}H] leucine (specific activity 122-146 Ci/mmol). Stored undiluted at 4°C.

L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]-arachidonyl (specific activity 56mCi/mmol). The stock solution was diluted to 10μCi/ml in toluene:ethanol (1:1) and stored at -20°C.

All radioactive compounds were supplied by Amersham International Ltd, Cardiff, U.K.

Antibodies	Source
Donkey anti-rabbit serum (DARS)	Scottish Antibody Production Unit, Carluke, Scotland
Normal rabbit serum (NRS)	
$PGF_{2\alpha}$ antiserum	Raised in this department in rabbits
PGE_2 antiserum	
6-keto-$PGF_{1\alpha}$ antiserum	

Gases

95% air and 5% CO_2

95% O_2 and 5% CO_2

All gases were supplied by British Oxygen Co. Ltd, Guildford, U.K.

Chromatography Materials	Source
PD-10 Desalting Columns	Pharmacia LKB Biotechnology, U.K.
Blue Sepharose CL-6B	Pharmacia LKB Biotechnology, U.K.
DEAE Sepharose CL-6B	Sigma Chemical Co., Poole, Dorset, U.K.
Sephadex G-75 SF	Pharmacia LKB Biotechnology, U.K.
Calibration Kit MW-GF-70	Pharmacia LKB Biotechnology, U.K.

Chromatography Equipment

All chromatography equipment was supplied by Pharmacia LKB Biotechnology Ltd, Milton Keynes, U.K.

Peristaltic Pump P-1

Fraction Collector Frac-100

Single-Channel Recorder REC-481

Single Path Monitor UV-1

Flow Cell HR 10

Gradient Mixer GM-1

Adaptor AC16

Column K15/40 (Dimensions 1.5cm x 40cm)

Column K16/40 (Dimensions 1.6cm x 40cm)

Column C16/100 (Dimensions 1.6cm x 100cm)

2:2 Composition of solutions

i. Krebs' Solution

This solution was used to maintain the guinea-pig uterus during superfusion experiments.

Sodium chloride	34.5g
Sodium hydrogen carbonate	10.5g
D-glucose	10.5g
Potassium chloride (10% w/v)	17.7ml
Magnesium sulphate (10% w/v)	14.5ml
Potassium dihydrogen orthophosphate (10% w/v)	8.0ml
Calcium chloride (1M)	12.6ml

Make up to 5l with distilled water and store at 4°C.

ii. Culture medium

Medium 199 was developed by Morgan, Morton and Parker (1950) as a

Table 1. The composition of Medium 199 (Modified) with Earle's salts and 2.2g/l sodium bicarbonate without glutamine.

Ingredient	mg/litre	Ingredient	mg/litre
L-Alanine	25.00	Menaptothone sodium bisulphate $3H_2O$	0.019
L-Arginine HCl	70.00	Nicotinic acid	0.025
L-Aspartic acid	30.00	Nicotinamide	0.025
L-Cysteine HCl	0.0987	p-Aminobenzoic acid	0.05
L-Cystine disodium salt	23.66	Pyridoxal HCl	0.025
L-Glutamic acid	66.82	Pyridoxine HCl	0.025
L-Glutamine	100.0	Riboflavin	0.01
Glutathione	0.05	Thiamin HCl	0.01
Glycine	50.00	DL-α Tocopherol phosphate disodium salt	0.01
L-Histidine HCl H_2O	21.88	Vitamin A acetate	0.1147
L-Hydroxyproline	10.00	$CaCl_2.2H_2O$	264.9
L-Isoleucine	20.00	$Fe(NO_3)_3.9H_2O$	0.10
L-Leucine	60.00	KCl	400.0
L-Lysine HCl	70.00	$MgSO_4.7H_2O$	200.0
L-Methionine	15.00	NaCl	6800
L-Phenylalanine	25.00	$NaHCO_3$	2200
L-Proline	40.00	$NaH_2PO_4.2H_2O$	158.3
L-Serine	25.00	Adenine sulphate	10.0
L-Threonine	30.00	5'AMP	0.20
L-Tryptophan	10.00	Cholesterol	0.20
L-Tyrosine disodium salt	49.72	2-Deoxyribose	0.50
L-Valine	25.00	D-Glucose	1000
L-Ascorbic acid	0.05	Guanine HCl	0.30
Biotin	0.01	Hypoxanthine	0.30
Calciferol	0.10	D-Ribose	0.50
D-Calcium pantothenate	0.01	Sodium acetate	36.71
Choline chloride	0.50	Phenol red sodium salt	17.00
Folic acid	0.01	Thymine	0.30
i-Inositol	0.05	Tween 80	5.00
		Uracil	0.30
		Xanthine	0.30

synthetic medium for the nutrition of animal cells in tissue culture. The medium is composed of amino acids, vitamins, nucleic acids and accessory growth factors supplemented with Earle's salts (Table 1).

Earle's salts are a synthetic mixture of inorganic salts known as a "physiological" or balanced salt solution. The functions of this salt solution in Medium 199 are:

1. To maintain the pH (sodium bicarbonate)
2. To maintain the osmotic pressure
3. To provide a source of energy (glucose)

Glutamine, which is an essential component for maintenance of tissue in culture (Fischer, Astrup, Ehrensvard and Oehlenschlager, 1948), is not stable above -10°C. It was therefore stored at -20°C and added to the culture medium when required. Antibiotic and fungicide were also added to prevent bacterial and fungal growth.

Medium 199 (plus Earle's salts)	500ml
Glutamine (200mM)	4ml
Amphoterecin B (Fungizone^{TM})(250μg/ml)	3ml
Kanamycin (5000μg/ml)	3ml

The medium was dispensed into 25ml storage bottles and stored at -20°C.

iii. <u>Phospholipase (PL)A_2 assay buffer</u>

300μl of this solution was used to incubate substrate [^{3}H]-phosphatidylcholine and the enzyme PLA_2 during PLA_2 assays.

Tris base	1.2g 0.1M
$CaCl_2$ (1M)	600.0μl 6mM
Disodium EDTA	44.0mg

Make up to 100ml with distilled water and adjust pH to 9.0 with 1M HCl. Store at 4°C.

iv. Radioimmunoassay solutions

a. $PGF_{2\alpha}$ diluent - 0.05M Tris buffer, pH 8.0

Tris base	30.25g
NaN_3	0.5g

Make up to 5l with distilled water and adjust to pH 8.0 with 1M HCl. To make up diluent, take 1l of Tris-buffer (pH 8.0) and add 1.0g gelatine (heat gently to dissolve). Store at 4°C.

b. 6-keto-$PGF_{1\alpha}$ diluent - 0.05M Tris buffer, pH 6.8

To make up diluent, take 1l of Tris-buffer (pH 8.0) and adjust pH to 6.8 with 1M HCl. Add 1.0g of gelatine. Store at 4°C.

c. PGE_2 diluent - Phosphate buffer, pH 7.5

Di-sodium hydrogen orthophosphate	34.5g
NaN_3	0.5g
Sodium dihydrogen orthophosphate (1M)	56.0ml

Make up to 5l with distilled water and adjust to pH 7.5 with 1M HCl. To make up diluent, take 1l of phosphate buffer (pH 7.5) and add 1.0g of gelatine. Store at 4°C.

d. Scintillation fluid

PPO	10.5g
2-Ethoxyethanol	900ml
Toluene	1500ml

v. Polyacrylamide gel electrophoresis stock solutions

Polyacrylamide gels are formed by co-polymerization of acrylamide and bisacrylamide (N,N'-methylene-bis-acrylamide). Polymerization is initiated by TEMED (tetramethylethylenediamine) and ammonium persulphate.

a. Matrix forming solutions

3% (w/v) polyacrylamide, 1mM NaN_3, 1mM NaF

NaN_3	6.5mg
NaF	4.2mg

Dissolve in 100ml distilled water and add 3g of polyacrylamide very slowly with vigorous stirring. Store at 4°C.

Acrylamide/bisacrylamide (30.8%T, 2.6%C)

Acrylamide	30.0g
Bisacrylamide	0.8g

Dissolve in 100ml distilled water and filter through glass wool. Store in the dark at 4°C.

NB %T refers to %w/v of total monomer (acrylamide+bis) in solution.

$$\%C = \frac{\text{gm crosslinker} \times 100}{(\text{gm monomer} + \text{gm crosslinker})}$$

b. Buffer Solutions

Resolving gel buffer

1.5M Tris-HCl, pH 8.8, 8mM EDTA, 0.4% SDS.

Tris base	90.85g
Disodium EDTA	1.50g
SDS	2.00g

Make up to 500ml with distilled water and adjust to pH 8.8 with 1M HCl. Store at 4°C.

Stacking gel buffer

1.5M Tris-HCl, pH 6.8, 8mM EDTA, 0.4% SDS.

Tris-base	3.0g
Disodium EDTA	0.15g
SDS	0.2g

Make up to 50ml with distilled water and adjust to pH 6.8 with 1M HCl. Store at 4°C.

Sample buffer (4-fold concentrated)

0.2M Tris-HCl, pH 6.8, 8mM EDTA, 8% SDS

Tris-base	2.42g
Disodium EDTA	0.29g
SDS	8.0g
Glycerol	40.0g

Make up to 100ml with distilled water and add 0.5ml of 0.5% (w/v) Bromophenol Blue (tracking dye). Store at 4°C.

Electrophoresis buffer (10-fold concentrated)

0.25M Tris-HCl, pH 8.3, 10mM EDTA, 0.5% SDS.

Tris-base	30.0g
Disodium EDTA	3.72g
SDS	5.0g
Glycine	144.0g

Make up to 1l with distilled water and adjust to pH 8.3 with 1M HCl. Store at 4°C.

2:3 Animals used

Guinea-pigs were housed in a 14 h light, 10 h dark cycle (lights on between 0500-1900) and received a diet of RGP pellets (Labsure,

Manea, U.K.), hay, vegetables and water supplemented with ascorbic acid. Oestrous cycles were monitored daily in female guinea-pigs (0.5-1.0kg) by examination of the vaginal membrane. A vaginal smear was taken when the membrane was open and the days of the cycle were numbered from the day before the post-ovulatory influx of leukocytes (Day 1) when maximum cornification was observed (Selle, 1922; Stockard and Papanicolaou, 1917; Nicol and Snell, 1954). Animals were only used after undergoing at least two normal oestrous cycles (about 17 days in length on average). Guinea-pigs were used on either Day-7 or Day-15 of the cycle (days of low and high $PGF_{2\alpha}$ output from the uterus respectively; Blatchley et al., 1972) or on Day-15 of pregnancy. Guinea-pigs were killed by stunning followed by rapid incision of the neck.

2:4 Superfusion of guinea-pig uterine horns

This technique was developed by Poyser and Brydon (1983) to measure the release of prostaglandins by the superfused guinea-pig uterus, using one horn as a control to measure basal PG output while the other is exposed to the test condition. Both uterine horns were removed and trimmed of any excess vascular or fatty tissue. Each horn was blotted dry, weighed and "opened" via a longitudinal incision before being suspended in an organ bath attached to an isotonic lever under a load of 2g. Each horn was superfused at a rate of 5ml/min with Krebs' solution, which had been pre-aerated with 5% CO_2 and 95% O_2, at 37°C.

After an initial "settling period" of 60 min, samples of superfusate from each uterine horn were collected for 10-min periods over the course of the experiment. After collection, the pH of each sample was lowered to 4.0 with the dropwise addition of 1M HCl. PGs

were extracted by shaking with two 50ml volumes of ethyl acetate. These 50ml fractions were combined and evaporated to dryness at 45°C on a rotary evaporator. Each extract was redissolved in 10ml of ethyl acetate and was stored at -20°C before the amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ present were measured by radioimmunoassay. This extraction procedure gives a high rate of recovery for $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$. The recoveries (mean $\pm$ s.e.m., $\underline{n}$=3) of the appropriate radioactive PGs at pH 4 were found to be 94$\pm$3% for $PGF_{2\alpha}$, 92$\pm$5% for PGE_2 (Poyser and Scott, 1980) and 82$\pm$2% for 6-keto-$PGF_{1\alpha}$ (Swan and Poyser, 1983). PG concentrations measured in samples of superfusates have not been corrected for these losses during recovery.

2:5 Culture of guinea-pig endometrium and conceptus tissue

Tissue was cultured by a method based on that described by Baker and Neal (1969) for ovarian tissue, and further refined for the culture of endometrial tissue by Abel and Baird (1980), Leaver and Seawright (1982) and Ning, Leaver and Poyser (1983).

The culturing of guinea-pig tissue was carried out for several differing purposes:

(i) Endometrium was cultured to measure PG output under various drug regimes.

(ii) Endometrium and conceptus tissue were cultured and the protein secreted into the culture medium was collected.

(iii) Endometrium was cultured with [^{3}H]-leucine to measure de novo secreted and cellular protein synthesis.

The longest culture period was 24h and histological examination has shown that guinea-pig endometrium remains viable during tissue culture over periods of up to 72h (Leaver and Seawright, 1982).

Method

i. Preparation of tissue and culture procedures

All experiments were carried out under strict aseptic techniques within a "Microflow Hood". All glass and plastic equipment was autoclaved prior to use and sterile culture dishes (5cm, vented) were used (Sterilin Ltd, Teddington, U.K.).

To culture endometrium, the uterus was removed from Day-7 or Day-15 guinea-pigs and, after separation, each horn was "opened" via a longitudinal incision. The endometrium was dissected from the myometrium by cutting away small pieces of endometrium with a pair of fine scissors. To culture conceptus tissue, the uterus was removed from Day-15 pregnant guinea-pigs, "opened" via a longitudinal incision and the conceptuses, including decidua, were gently removed. The tissue (endometrium or conceptus) was cut into small (1-2mm^3) pieces and placed on a section of sterile lens tissue which was lying across a raised stainless steel platform in a sterile petri dish with its ends dipping into 4ml of culture medium. Thus the tissue was not immersed in the medium but had access to it via capillary action through the lens tissue (Figure 3). The culture medium consisted of Medium 199 plus Earle's salts, supplemented with glutamine, amphotericin B (Fungizone^{TM}) and kanamycin. In certain culture experiments in which the effect of proteins purified from guinea-pig endometrium and conceptus was tested on the production of prostaglandins from Day-15 endometrium, smaller petri dishes (Lux 35 x 10mm, Flow Laboratories, Irvine, U.K.) containing only 2ml of culture medium were used in order to maximise the concentration of protein in the medium. Once all the tissue had been cut up and laid on the lens tissue, the petri dishes were stacked in a steel rack and placed in a modified Kilner jar. Each jar was pressurised to

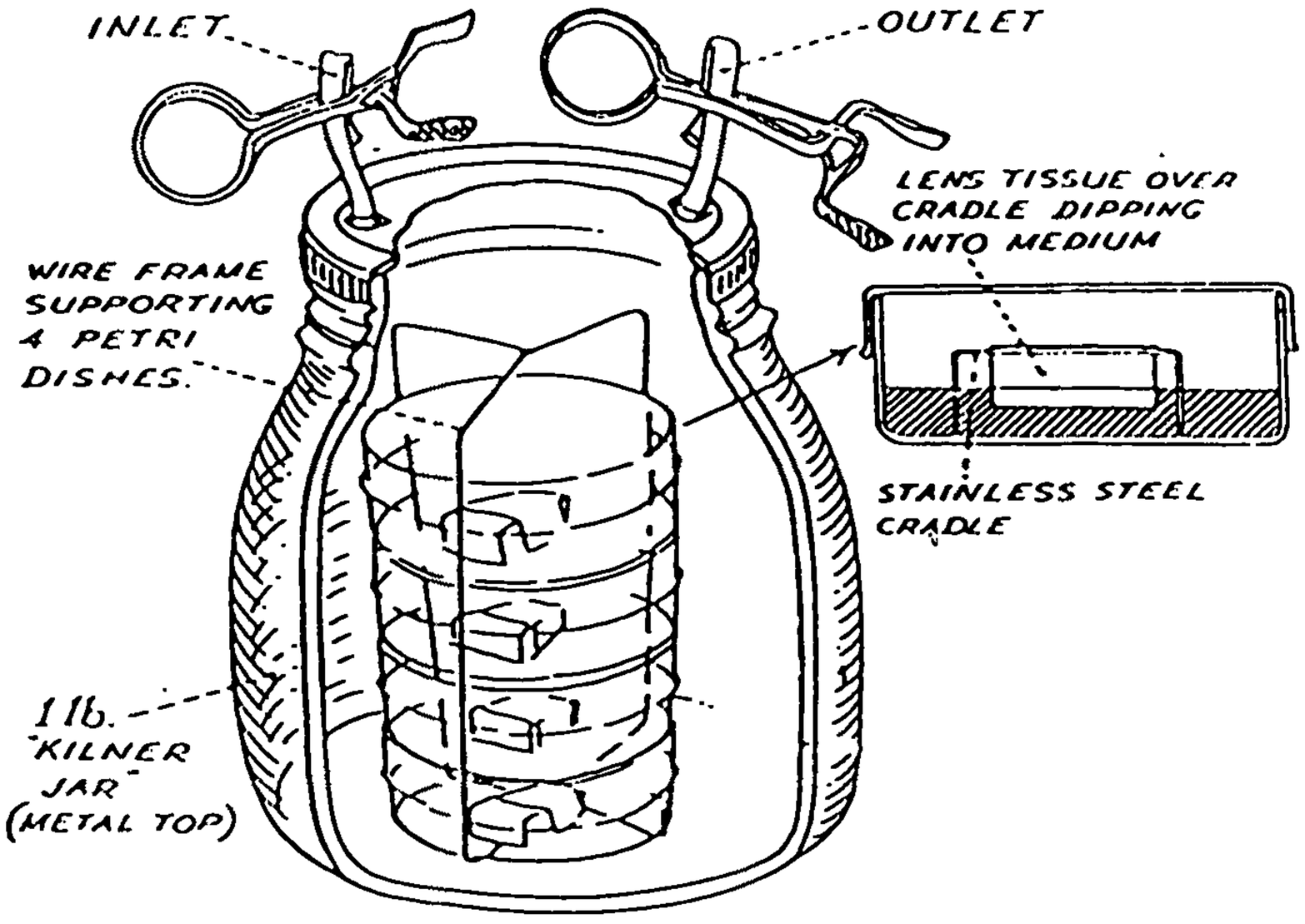


Fig. 3. The above diagram represents the equipment used to culture guinea-pig endometrium and conceptus tissue.

0.7kg/cm^2 (10lb/in^2) with a 1:1 mixture of air/CO_2 (95%:5%) and O_2/CO_2 (95%:5%) and incubated at 37°C for the course of the experiment. The jars were regassed if removal and replacement of medium was carried out during the experiment. Fresh medium was prewarmed to 37°C.

ii. Collection of medium

If the medium was to undergo analysis for prostaglandin content, it was removed from each dish separately into plastic tubes (Sterilin Ltd., Teddington, U.K.) and stored at -20°C until assayed. The tissue from each dish was removed separately, dried in an oven at 37°C for 24 h and weighed.

If the medium was being collected for protein purification, it was removed and pooled for dialysis at 4°C. The tissue from each animal was amalgamated, dried in an oven at 37°C for 24 h and weighed.

If measurement of the amounts of [^{3}H]-leucine incorporated into cellular and secreted proteins was to be carried out, the medium was removed from each dish and immediately centrifuged before carrying out the analysis. The tissue was removed from each dish separately, weighed and washed in culture medium at 4°C.

2:6 Measurement of [^{3}H]-leucine incorporation into cellular and secreted proteins in guinea-pig endometrium

Introduction

This technique was used to investigate protein synthesis by guinea-pig endometrium maintained in culture. Measurement of [^{3}H]-leucine incorporation into proteins indicates how much *de novo* protein synthesis has occurred during the culture period. The methods used are summarised in Figure 4.

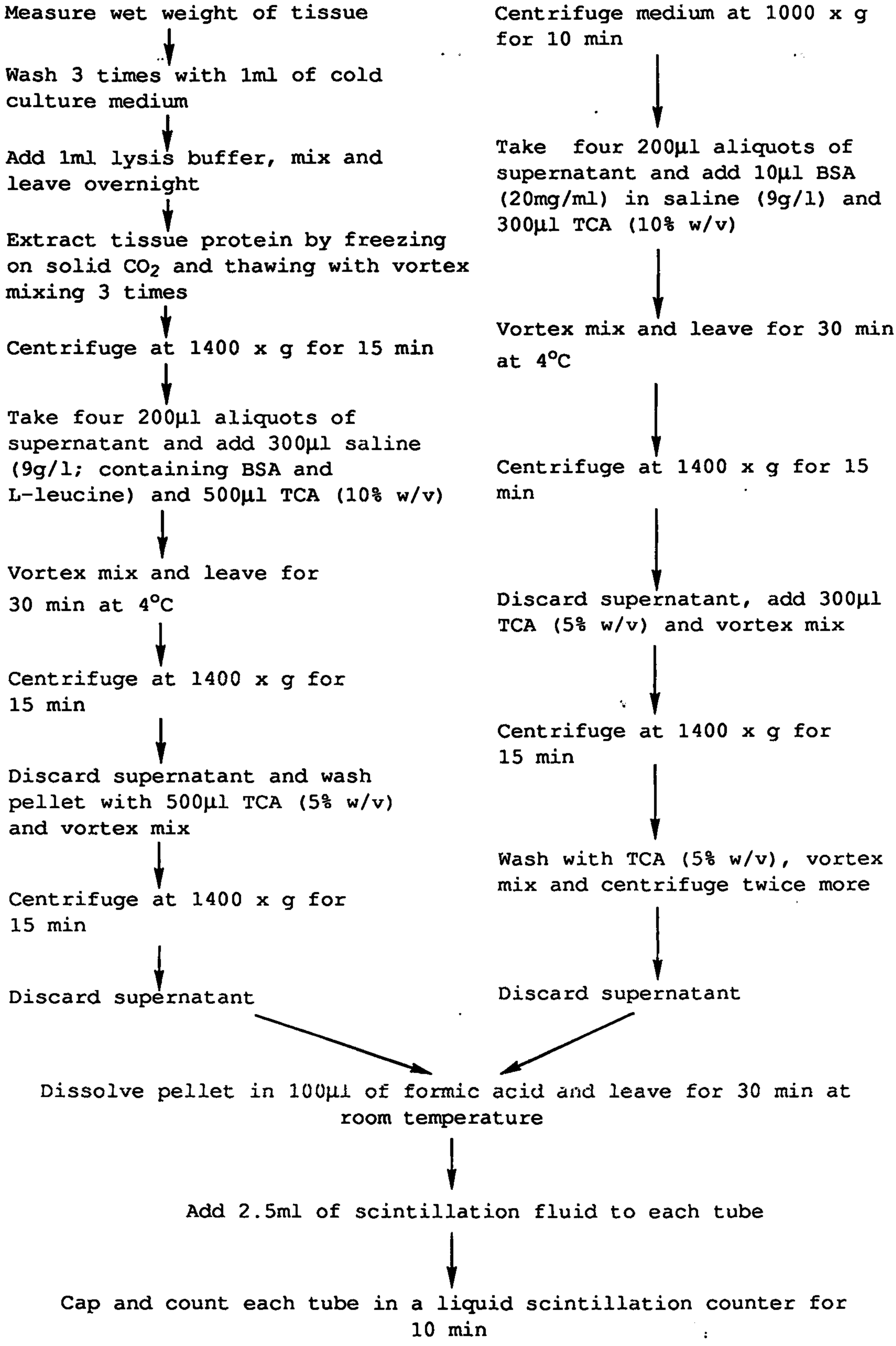


Fig. 4. The methods used for measuring [^{3}H]-leucine incorporation into cellular and secreted proteins.

Method

i. Cellular protein

This technique was based on the method of Findlay, Ackland, Burton, Davis, Maule Walker, Walters and Heap (1981) and developed for guinea-pig endometrium by Riley and Poyser (1989). Endometrium (25-30mg) was cultured for 24h in the presence of [^{3}H]-leucine (10μCi per dish). The tissue was then removed, weighed, and washed 3 times with 1ml of culture medium at 4°C. The tissue was placed in 1ml lysis buffer (8M urea, 1% w/v sodium dodecyl sulphate and 5% v/v mercaptoethanol), shaken and left at 4°C overnight. Tissue proteins were extracted by freezing the tissue on solid CO_2 followed by thawing with vortex mixing. This procedure was performed 3 times. The samples were then centrifuged at 1400 x g for 15 min and four 200μl volumes of supernatant were mixed with 300μl saline (9g/l) containing bovine serum albumin (0.1% w/v) and L-leucine (0.1% w/v) as carriers. Proteins were precipitated by adding 500μl of trichloroacetic acid (TCA) (10% w/v) and allowed to stand for 30 min at 4°C. Each sample was centrifuged at 1400 x g for 15 min and the supernatant was discarded. The precipitate was washed with 500μl TCA (5% w/v), vortex mixed to resuspend the precipitate and centrifuged at 1400 x g for 15 min. The supernatant was again discarded and the precipitate was dissolved by incubating in 100μl formic acid for 30 min at room temperature. 2.5ml of scintillation fluid were added to each sample, the tubes were capped and the amount of radioactivity present in each tube was measured by counting for 10 min in a Canberra Packard Tricarb 4000 Series liquid scintillation counter.

ii. Secreted protein

This technique was based on the method of Strinden and Shapiro

(1983) and developed for use on the guinea-pig endometrium by Riley and Poyser (1989). Endometrium (25-30mg) was cultured for 24h in the presence of [^{3}H]-leucine (10μCi per dish). The samples of culture medium from each dish were removed and centrifuged at 1000 x g for 10 min. To four 200μl volumes of the supernatant, 10μl of a solution containing bovine serum albumin (20mg/ml) in saline (9g/l) and 300μl of TCA (w/v 10%), were added. After incubation for 30 min at 4°C, each sample was centrifuged at 1400 x g for 15 min. The supernatant was discarded and the precipitate was washed with 300μl TCA (w/v 5%). The tubes were vortex mixed and centrifuged at 1400 x g for 15 min. This procedure was repeated 3 times to remove any free [^{3}H]-leucine, and finally the precipitate was dissolved by incubating with 100μl formic acid for 30 min at room temperature. 2.5ml of scintillation fluid were added to each sample and the amount of radioactivity present in each tube was measured by counting in a Canberra Packard Tricarb 4000 Series liquid scintillation counter for 10 min.

iii. Controls:- Non-specific binding measurements

Immediately after the jar containing the cultured tissue was placed in the incubator, 30-60mg of endometrium were placed in another petri dish containing 10μCi of [^{3}H]-leucine. The endometrium was rinsed in the culture medium and left for 2 min at room temperature before being removed and weighed. The endometrium and culture medium were then subjected to the procedures for measuring the amounts of [^{3}H]-leucine incorporated into cellular and secreted proteins respectively, in order to measure the amounts of [^{3}H]-leucine which were bound non-specifically.

2.7 Phospholipase A_2 assay

This assay was based on a method developed by Consentino and Ellis (1981) in which the metabolites and substrates were eluted from silica gel columns with two different solvent systems so that each could be quantified separately. The following procedure incorporates modifications made by Downing and Poyser (1983).

Method

i. Incubation procedure

Phosphatidylcholine (PC) with [^{14}C]-arachidonic acid ([^{14}C]-AA) in the 2-position was used as a labelled substrate for this assay. The radioactive substrate was stored in a solution of toluene/methanol (1:1) which was evaporated under a stream of N_2 before the residue was dissolved in distilled water by sonicating at 4°C for 30 min. 300μl of PLA_2 assay buffer consisting of 0.1M Tris-HCl, 6mM $CaCl_2$ and 0.0015M EDTA at pH 9.0, and containing 0, 0.2, 0.5, 1.0 or 2.0 U/ml PLA_2 from Naja naja venom, was dispensed into Eppendorf tubes with each concentration of PLA_2 being used in triplicate. The reaction was started by adding 0.2μCi (approximately 10,000 c.p.m.) of labelled substrate in 20μl to each sample, and the incubation was carried out for 10 min at 37°C. The reaction was stopped by adding 20μl of 2M HCl, and 10μl arachidonic acid solution (1mg/ml in ethanol) were added to act as a carrier for [^{14}C]-AA released from the labelled substrate. The incubates were frozen at -20°C and stored overnight.

ii. Separation of [^{14}C]-PC and [^{14}C]-AA

Pasteur pipettes were plugged with teflon wool and packed to a height of 3cm with silica gel (100-200 mesh) suspended in

hexane/dioxan/glacial acetic acid (90:10:1) (Solvent 1). The column was washed with 1ml of Solvent 1 before adding the thawed incubate. The fatty acid ([^{14}C]-AA) was eluted by five 1ml fractions of Solvent 1 and the unmetabolised substrate ([^{14}C]-PC) was eluted by seven 1ml fractions of chloroform/ methanol/water (65:35:4) (Solvent 2). Each 1ml fraction was collected in a mini scintillation vial and the solvent was evaporated off in a heating block at 45°C under a stream of air. Scintillation fluid (2.5 ml) was added to each vial. The radioactivity present was measured by counting in a Canberra Packard, Tricarb 4000 series scintillation counter for 4 min. The activity of PLA_2 was measured as the conversion of [^{14}C]-PC to [^{14}C]-AA during a 10 min incubation period calculated as a percentage of the total radioactivity recovered.

iii. Results and conclusions

The results of incubating varying concentrations of PLA_2 with a fixed concentration of phosphatidylcholine are shown in Figure 5. Maximum activity of the enzyme occurred at a concentration of 1.0 U/ml. A concentration of 0.5 U/ml of PLA_2 was used when determining the effects of proteins purified from guinea-pig endometrium or conceptus, on the activity of PLA_2 as this concentration of the enzyme gave a conversion of [^{14}C]-PC to [^{14}C]-AA of approximately 65% and therefore could be used to detect either inhibition or stimulation of the activity of PLA_2.

To assay the effect of endometrial or conceptus proteins on the activity of PLA_2, 1mg of each protein were dissolved in 1ml of PLA_2 assay buffer containing 0.5U/ml of PLA_2. 300μl of each protein solution were dispensed into Eppendorf tubes in triplicate and 3 controls containing no protein at the same concentration of

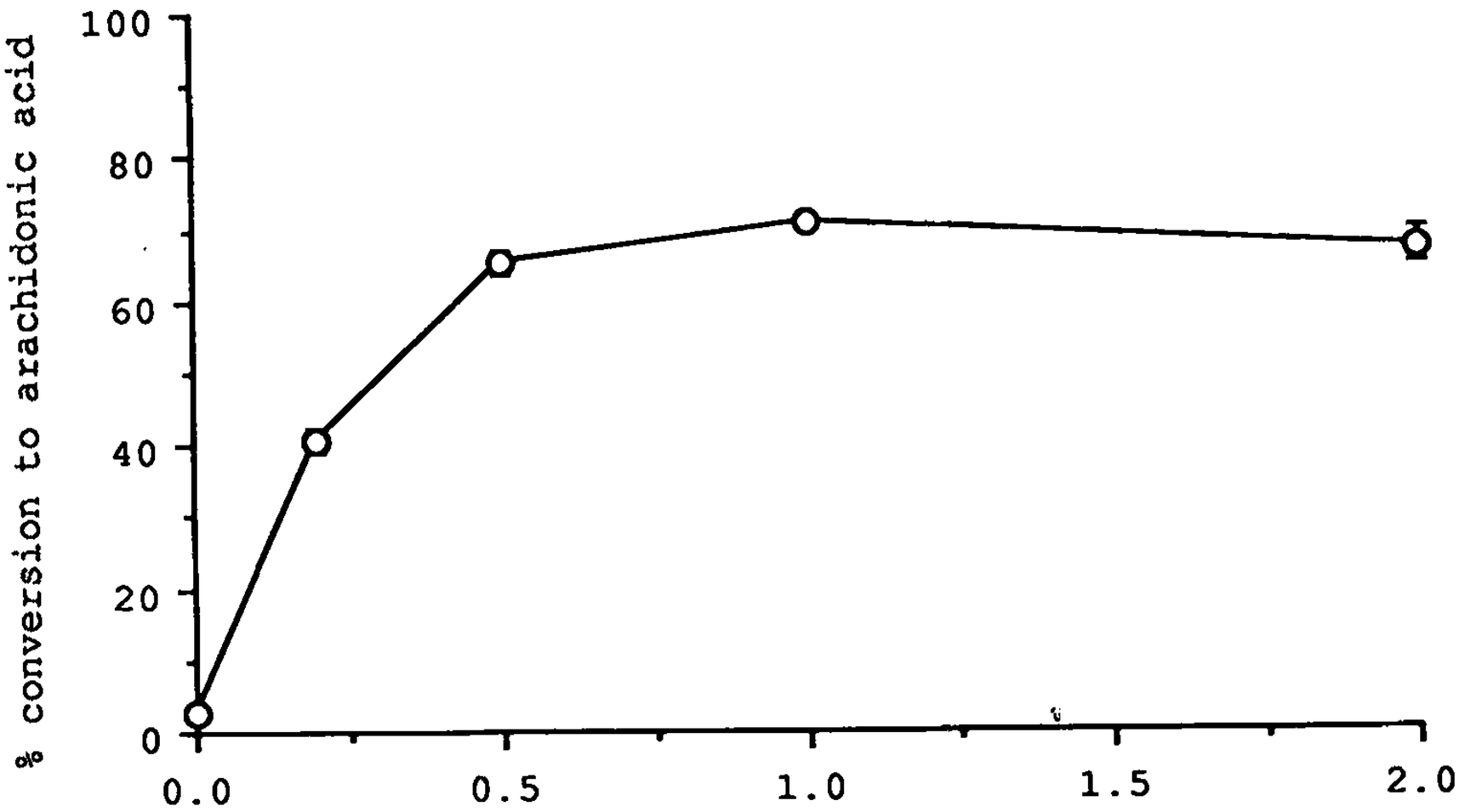


Fig. 5. Mean ± s.e.m. % conversion (n = 3) of [^{14}C]-phosphatidylcholine to [^{14}C]-arachidonic acid at varying concentrations of phospholipase A_2 and a fixed concentration of substrate (20μM).

PLA_2 were also set up for each assay.

2:8 Polyacrylamide gel electrophoresis (PAGE)

Introduction

Polyacrylamide gel electrophoresis (PAGE) in the presence of the anionic detergent sodium dodecyl sulphate (SDS) was first used to identify and separate polypeptide chains (Maizel, 1966; Shapiro, Scharff, Maizel and Uhr, 1966; Vinuela, Algranati and Ochoa, 1967). However, Shapiro, Vinuela and Maizel (1967) noticed that protein migration was inversely related to the logarithm of protein molecular weight on SDS-gel electrophoresis, and so this technique could be used for the rapid and simple estimation of the molecular weight of proteins and their subunits. Weber and Osborn (1969) and others (Dunker and Rueckert, 1969; Neville, 1971) verified that the molecular weights obtained by SDS-gel electrophoresis were reproducible and reliable. Reynolds and Tanford (1970) found that the interaction of the detergent sodium dodecyl sulphate (SDS) with a wide variety of proteins was characterised by a high level of binding of SDS to proteins and a similar binding ratio on a gram to gram basis. This resulted in a constant charge per unit mass, minimising the native protein charge differences and leading to all proteins exhibiting highly anionic behaviour. Thus the migration of protein-SDS complexes on polyacrylamide gels depended on the unique function of polypeptide chain length alone under any given set of electrophoretic conditions (i.e. pH, voltage gradient, time, gel concentration).

The resolution of ions based on dimensional differences was found to be increased by the introduction of an electrophoretic step for concentrating the sample ions into a narrow starting zone prior to

electrophoretic separation (Ornstein, 1964; Davis, 1964). This technique known as disc electrophoresis was adapted for use in protein electrophoresis by Laemmli (1970). This discontinuous system was capable of performing size separation of proteins with high resolution (Neville, 1971; Neville and Glossmann, 1971; King and Laemmli, 1971).

Proteins obtained from tissue culture medium and separated and purified by affinity, ion-exchange and gel filtration chromatography had their molecular weights determined by SDS-polyacrylamide gel electrophoresis on homogenous gels using a discontinuous buffer system. To determine the molecular weights of unknown proteins, their electrophoretic mobilities were compared with the electrophoretic mobilities of protein standards of known molecular weight.

Method

In discontinuous systems, a resolving gel is polymerised first. Temperature affects the properties of the gel so all stock solutions, which were stored at 4°C, were equilibrated to room temperature before use. The following reagents were mixed in a 100ml round-bottomed flask.

i. Resolving gel preparation (12%)

3%(w/v) polyacrylamide, 1mM NaN_3, 1mM NaF	5.0g
Acrylamide/bisacrylamide (30.8%T, 2.6%C)	12.0ml
Resolving gel buffer (pH 8.8)	7.5ml
Distilled water	5.2ml
TEMED	10.0μl

The mixture was then degassed for at least 15 min (a process which

is faster and more complete if the gel solution is at room temperature). Meanwhile the gel casting mould was prepared by inserting spacers between two glass plates and clamping them together onto a gel casting stand (all equipment supplied by Bio-Rad Laboratories Ltd, Watford, U.K. as part of the PROTEAN II™ Vertical Electrophoresis System).

A 10% ammonium persulphate solution was freshly prepared and 200μl were added to the degassed gel solution to initiate polymerisation. The solution was swirled gently to mix the initiators and poured into the gel mould in a steady stream in order to minimise the introduction of air bubbles. The gel was gently overlaid with 1ml of a four-fold dilution of resolving gel buffer in order to exclude oxygen (which inhibits polymerization) from the surface. Polymerization was allowed to occur for at least 90 min. During this time the stacking gel was prepared in a 100ml round-bottomed flask.

ii. Stacking gel preparation (4.6%)

3%(w/v) polyacrylamide, 1mM NaN_3, 1mM NaF	1.3g
Acrylamide/bisacrylamide (30.8%T, 2.6%C)	1.2ml
Stacking gel buffer (pH 6.8)	2.0ml
Distilled water	3.4ml
TEMED	5.0μl

This solution was degassed for at least 15 min before adding 50μl of fresh 10% ammonium persulphate with swirling. The buffer overlay was removed from the lower resolving gel and 1ml of "initiated" stacking gel was used to rinse the top of the gel and then removed. The remainder of the stacking gel was then poured into the gel mould and a well-forming comb was carefully inserted into the gel to avoid trapping air under the teeth. Polymerization was allowed to occur for at least 45 min.

iii. Sample preparation

The low molecular weight (LMW) calibration kit (supplied by Pharmacia LKB Biotechnology Ltd, Milton Keynes, U.K.), containing six protein standards covering the molecular weight range from 14,000 to 94,000 Da, was used for molecular weight determination (Table 2).

The exact amounts of each protein (approximately 100μg) were chosen to give bands of equal intensity on staining with Coomassie Blue. The protein standard mixture was dissolved in 100μl of Sample Buffer (4-fold diluted). 100μg of each unknown protein was weighed out and dissolved in 100μl of Sample Buffer (4-fold diluted). Once the proteins were dissolved, they were heated at 100°C for 5 min. Meanwhile 1.5l of electrophoresis buffer was made up by making a 1 in 10 dilution of stock electrophoresis buffer. The well-forming comb was removed from the set gel, which was now clamped onto the central cooling core. 1,150ml of electrophoresis buffer was placed in the tank and the cooling core, with the gel clamped on one side and an acrylic plate on the other, was lowered into the tank at an angle to displace any air bubbles from under the gel sandwich. The upper chamber was filled with the remaining 350ml of electrophoresis buffer.

iv. Loading of samples

10μl of molecular weight standard solution or unknown protein solution were loaded into each well using a Hamilton^{TM} micro-syringe. The presence of glycerol in the sample buffer increased the density of the protein samples and allowed loading to be carried out under the electrophoresis buffer. Wells filled with molecular weight standard proteins (2-3) were distributed along the

Table 2. The composition of proteins in each vial in the Low Molecular Weight Electrophoresis Calibration Kit.

Protein	Source	Molecular Weight
Phosphorylase b	rabbit muscle	94,000
Albumin	bovine serum	67,000
Ovalbumin	egg white	43,000
Carbonic anhydrase	bovine erythrocyte	30,000
Trypsin inhibitor	soybean	20,100
α-Lactalbumin	bovine milk	14,400

gel to account for any inconsistencies in the gel.

50mA of current was applied to the gel until the samples had entered into the stacking gel. The gel was then allowed to run overnight (approximately 16 h) at 10mA until the tracking dye migrated to within 1-2 cm of the bottom of the gel.

v. Fixing, staining and destaining the gel

After completion of electrophoresis, the gel was removed from its mould and was placed in 300ml of fixing solution (10% w/v trichloroacetic acid and 20% v/v methanol) for 30 min. The gel was then stained with Coomassie Blue by incubating at 37°C in a shaking water bath for 30 min in a 300ml solution of 7.5% v/v glacial acetic acid, 50% v/v methanol and 0.25% w/v Coomassie Blue-R. After staining, the gel was washed with distilled water to remove any unbound dye. Destaining was carried out by incubating the gel with a 300ml solution of 7% v/v acetic acid and 10% methanol for 1h at 37°C in a shaking water bath. The destaining process was repeated 2-3 times until the gel matrix was completely dye-free and only the proteins present in the gel were coloured blue.

vi. Molecular weight determination

In order to measure the molecular weights of the unknown protein samples, a calibration curve was made for the protein standards by calculating the relative migration value (Rf) for each protein. This was done by measuring the migration distance of each protein standard on the stained gel and the migration distance of the dye front (used as a reference point).

Rf was defined as $\frac{\text{distance protein migrated from origin}}{\text{distance from origin to dye front}}$

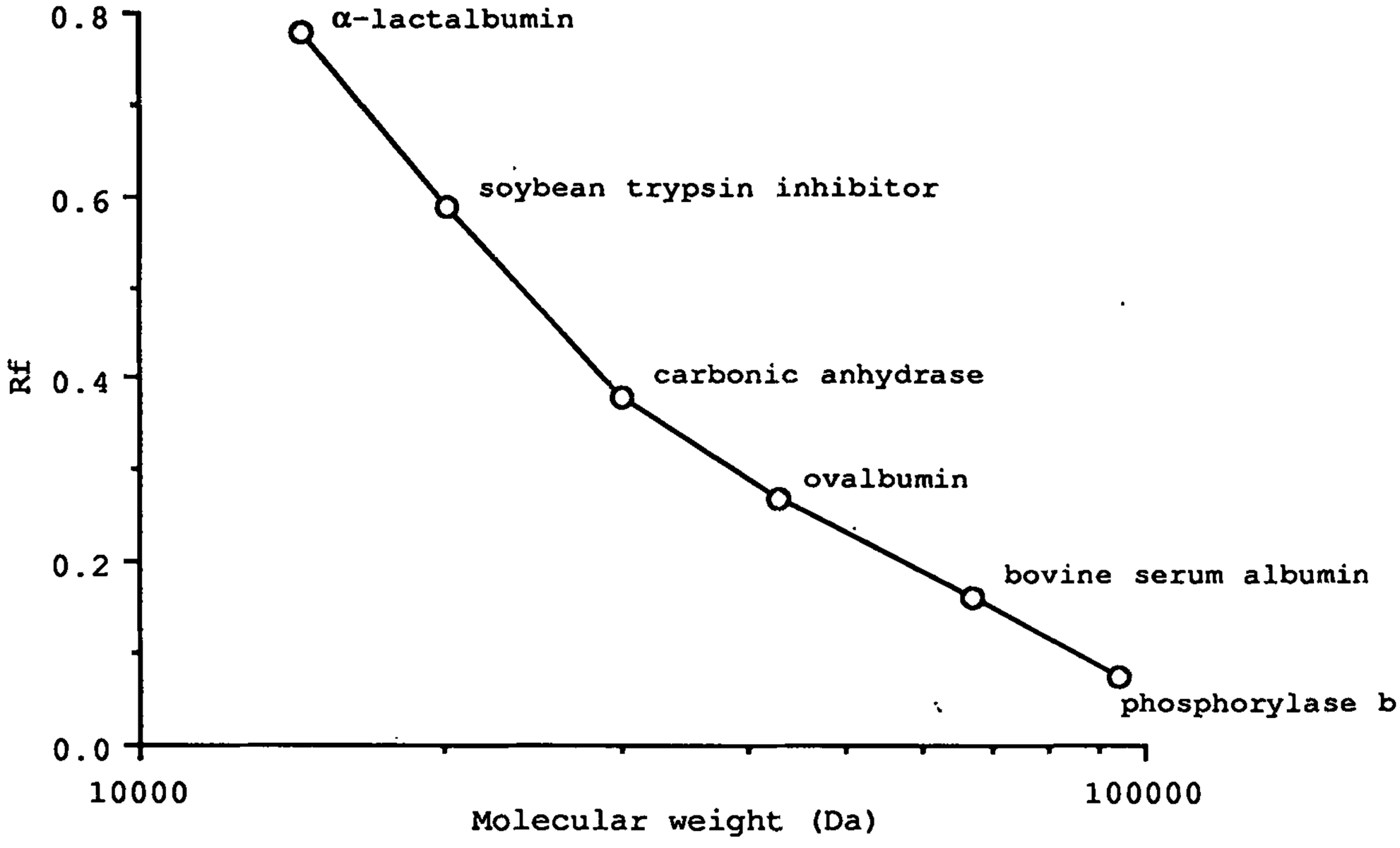


Fig. 6. Standard curve for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) obtained with proteins from the Pharmacia Low Molecular Weight Calibration Kit. Each point is the mean ± s.e.m. (n = 3), although, the size of the points are greater than the standard error. Rf = the relative migration value.

A calibration curve was constructed by plotting the Rf values for protein standards versus the logarithms of their corresponding molecular weights. The molecular weights of the unknown proteins were measured by calculating the Rf values and locating the point on the calibration curve which corresponds to that value. Rf values were only compared between standards and unknowns run on the same gel. Figure 6 shows the calibration curve obtained from the combined result (mean ± s.e.m.) of running three standard protein samples on the same gel.

2:9 Measurement of prostaglandins by radioimmunoassay

Introduction

Radioimmunoassay is a powerful technique developed in the 1960's. It combines the specificity of antigen-antibody reactions with the sensitivity of radioisotopic detection. The concentration of the unknown unlabelled antigen is obtained by comparing its inhibitory effect on the binding of radioactively labelled antigen (tracer) to a specific antibody with the inhibitory effect of known standards. Bound antigen is separated from unbound antigen by precipitating the antigen-antibody complexes with antiserum raised against the antibody. This is known as the double antibody method.

Method

i. Preparation of standards

Assay tubes 1-38 of the $PGF_{2\alpha}$ assay and 1-35 of the PGE_2 and 6-keto-$PGF_{1\alpha}$ assays contain standard solutions. Tubes 1-4 measure "non-specific binding of radioactive tracer" to the antibody which should be less that 10% for a valid assay. Tubes 5-8 are the "counting standards" which contain only radioactive tracer. Tubes

9-35 or 9-38 consist of nine (for the 6-keto-$PGF_{1\alpha}$ and PGE_2 assays) or ten (for the $PGF_{2\alpha}$ assay) standard concentrations respectively, each of which is dispensed out in triplicate, and are used to construct the standard curve (Table 3).

Stock prostaglandin solutions were stored in methanol at -20°C at a concentration of 1μg/ml. 200μl of standard were removed and the methanol was blown off under a stream of air. The prostaglandin was resuspended in 20ml of the appropriate diluent to give a 10ng/ml solution. 0.5ml of this solution was placed in each of the first 4 tubes of the assay to measure non-specific binding (i.e. the concentration of unlabelled PG is high enough to prevent labelled PG binding to any specific PG binding sites on the antibody and any binding is non-specific). The next prostaglandin concentration was made up by taking 10.24ml of the 10ng/ml solution and adding 9.76ml of diluent. This gave a 5.12ng/ml solution. The other standards were prepared by making serial 2-fold dilutions of the 5.12ng/ml solution down to 0.005ng/ml for the $PGF_{2\alpha}$ assay and 0.02ng/ml for the 6-keto-$PGF_{1\alpha}$ and PGE_2 assays. The 5.12ng/ml concentration of $PGF_{2\alpha}$ was not used in constructing the $PGF_{2\alpha}$ standard curve as a maximum of 10 standard solutions could be used to calculate the standard curve by the Spline Curve-Fit programme on the IBM-personal computer linked to the scintillation counter. The standards were stored at -20°C. The last four tubes of the assay contained 500μl diluent in duplicate (zero standards) and 500μl of the 0.32ng/ml standard solution in duplicate (for calculation of inter-assay coefficients of variation).

ii. <u>Preparation of radioactive tracers</u>

The amount of [^{3}H]-PG used in each assay was calculated to give

Table 3. The concentration and volume of standard solutions of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 used to construct each radioimmunoassay standard curve.

	Concentration of standard solution (ng/ml)		
Tube no.	$PGF_{2\alpha}$	PGE_2/ 6-keto-$PGF_{1\alpha}$	Volume(μl)
1-4	10.0	10.0	500
5-8	-	-	-
9-11	0.005	0.02	500
12-14	0.01	0.04	500
15-17	0.02	0.08	500
18-20	0.04	0.16	500
21-23	0.08	0.32	500
24-26	0.16	0.64	500
27-29	0.32	1.28	500
30-32	0.64	2.56	500
33-35	1.28	5.12	500
36-38	2.56	-	500

between 15,000-20,000 counts per tube when counted in a scintillation counter for 4 min ([^{3}H]-$PGF_{2\alpha}$ and [^{3}H]-6-keto-$PGF_{1\alpha}$) or for 10 min ([^{3}H]-PGE_2]). The radioactive tracer prostaglandins were stored at a concentration of 5μCi/ml in methanol, except [^{3}H]-6-keto-$PGF_{1\alpha}$ which was stored in acetonitrile:water (9:1), at -20°C. Prior to use, the solvent carrier was evaporated off under a stream of air, and [^{3}H]-$PGF_{2\alpha}$ was diluted to 0.125-0.17μCi/ml, [^{3}H]-PGE_2 was diluted to 0.05-0.0625μCi/ml and [^{3}H]-6-keto-$PGF_{1\alpha}$ was diluted to 0.17-0.2μCi/ml in the appropriate diluent.

iii. Preparation of antibodies

The dilution of antiserum chosen was that which bound 60% of tracer PG in the absence of non-radioactive standard PG. Antibodies to $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were stored at 100-fold dilutions in the appropriate diluent at -20°C. Antibodies to PGE_2 were stored undiluted at -20°C. Each antibody was diluted to the appropriate concentration immediately prior to use. (Final dilution 1 in 1200 for $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ antisera and 1 in 150 for PGE_2 antisera). Donkey anti-rabbit serum (DARS) and normal rabbit serum (NRS) were stored at -20°C and diluted immediately prior to use. DARS was used 10-fold diluted for all assays and NRS at a 100-fold dilution (except for PGE_2 assays where no NRS was used). NRS was added to ensure that, at high dilutions of the antiserum, there was sufficient gamma-globulin present for adequate precipitation of bound prostaglandins. It was not necessary to add NRS to the PGE_2 assay as a low dilution of PGE_2 antiserum was used. DARS was added to separate free PG from antibody-bound PG.

iv. Cross-reactivities of antisera

a. $PGF_{2\alpha}$

Antisera to $PGF_{2\alpha}$ were raised in this department in rabbits immunised with $PGF_{2\alpha}$ conjugated to bovine serum albumin (BSA) using the procedure described by Dighe, Emslie, Henderson, Rutherford and Simon (1975). The percentage cross reactivity of the antiserum was determined by finding the concentration of PG or PG metabolite which produced a given fall in binding with the zero standard and comparing it with the concentration of $PGF_{2\alpha}$ which produced an identical fall in binding with the zero standard.

$$\% \text{ cross reactivity} = \frac{\text{concentration of } PGF_{2\alpha} \text{ giving a fall in zero-binding} \times 100}{\text{concentration of PG or PG metabolite giving an identical fall in zero binding}}$$

The cross-reactivities as determined previously (Dighe et al., 1975; Poyser and Scott, 1980; Lytton and Poyser, 1982a) are shown in Table 4. The only significant cross reactivity of the $PGF_{2\alpha}$ antiserum used was with $PGF_{1\alpha}$. However analysis by gas chromatography and mass spectrometry has previously shown that the amount of $PGF_{1\alpha}$ present in extracts of guinea-pig uterus and endometrium is only about 1-2% of the amount of $PGF_{2\alpha}$ present (Poyser, 1983a). Therefore the $PGF_{2\alpha}$ antiserum will measure predominantly $PGF_{2\alpha}$ from guinea-pig uterus and endometrium.

b. PGE_2

Antisera to PGE_2 were raised in this department in rabbits immunised with PGE_2 conjugated to thyroglobulin using the

Table 4. The cross reactivities of $PGF_{2\alpha}$ antiserum from rabbit 6, 6th bleed with various prostaglandins and their metabolites at 30% binding with tracer.

Prostanoid	% cross reactivity
$PGF_{2\alpha}$	100
$PGF_{1\alpha}$	28
PGE_2	0.17
PGE_1	0.54
PGA_2	0.04
PGB_2	0.01
PGD_2	0.38
15-keto-$PGF_{2\alpha}$	0.45
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.21
15-keto-PGE_2	0.01
13,14-dihydro-15-keto-PGE_2	0.01
6-keto-$PGF_{1\alpha}$	0.47
TXB_2	0.58

procedure described by Dighe, Smith, Ungar and Whelpdale (1978). The cross reactivities as determined previously (Poyser and Scott, 1980; Lytton and Poyser, 1982a) are shown in Table 5. The antiserum had a very high cross reactivity with PGE_1 and significant cross reactivity with PGA_2 and PGB_2. However PGE_1, PGA_2 and PGB_2 could not be detected in extracts of guinea-pig uterus, endometrium, myometrium or ovary after analysis by gas chromatography and mass spectrometry (Poyser, 1983a). Therefore the PGE_2 antiserum is specific for measuring PGE_2 in guinea-pig reproductive tissues.

c. <u>6-keto-$PGF_{1\alpha}$</u>

Antisera to 6-keto-$PGF_{1\alpha}$ were raised in this department in rabbits immunised with 6-keto-$PGF_{1\alpha}$ conjugated to thyroglobulin using the procedure prescribed by Dighe, Jones and Poyser (1978). The cross-reactivities as determined previously (Poyser and Scott, 1980) are shown in Table 6. PGE_2, PGE_1 and $PGF_{1\alpha}$ were the only substances found to cross react with 6-keto-$PGF_{1\alpha}$ antiserum with any significance but the levels were considered low enough (<5%) to enable use of the antiserum for specifically measuring 6-keto-$PGF_{1\alpha}$.

v. <u>Assay procedure</u>

Samples to be assayed for PG content were dispensed in duplicate in a volume appropriate to allow the binding to lie on the steepest part of the standard curve for the particular PG being assayed. Samples obtained from superfusion experiments, which had been stored in ethyl acetate, were dispensed in duplicate at an appropriate volume and the ethyl acetate was evaporated off under a stream of air at 45°C in a heating block. 500μl of the appropriate diluent

Table 5. The cross reactivities of PGE_2 antiserum from rabbit 5, 6th bleed with various prostaglandins and their metabolites at 50% binding with tracer.

Prostanoid	% cross reactivity
PGE_2	100
PGE_1	66
PGA_2	26
PGB_2	12
$PGF_{2\alpha}$	1.5
PGD_2	0.4
15-keto-$PGF_{2\alpha}$	0.1
13,14-dihydro-15-keto-$PGF_{2\alpha}$	<0.1
15-keto-PGE_2	0.2
13,14-dihydro-15-keto-PGE_2	0.4
TXB_2	0.2

Table 6. The cross reactivities of 6-keto-$PGF_{1\alpha}$ antiserum from rabbit NP1, 6th bleed with various prostaglandins and their metabolites at 30% binding with tracer.

Prostanoid	% cross reactivity
6-keto-$PGF_{1\alpha}$	100
$PGF_{1\alpha}$	0.43
PGE_2	4.2
PGE_1	1.1
PGA_2	0.07
PGB_2	0.03
$PGF_{2\alpha}$	0.01
PGD_2	0.01
15-keto-$PGF_{2\alpha}$	0.04
13,14-dihydro-15-keto-$PGF_{2\alpha}$	0.07
15-keto-PGE_2	0.08
13,14-dihydro-15-keto-PGE_2	0.09
TXB_2	<0.01

were then added to each sample. Samples obtained from tissue-culture experiments were allowed to thaw before being dispensed out in duplicate at an appropriate volume. An equal volume of culture medium was added to each of the prostaglandin standards in order to account for any changes in binding caused by the culture medium. 500μl of the appropriate diluent were then added to each sample. Radioactive tracer (50μl) was added to all tubes using a Hamilton^{TM} glass syringe contained in an automatic dispenser. 50μl of antiserum were then added to all tubes except the counting standards. After vortex mixing, the tubes were left to incubate for 1h for the $PGF_{2\alpha}$ assay or 2h for the PGE_2 and 6-keto-$PGF_{1\alpha}$ assays at room temperature. 50μl of normal rabbit serum (NRS) and 50μl of donkey anti-rabbit serum (DARS) were added to all tubes except the counting standards. After vortex mixing, the tubes were incubated overnight (16h) at 4°C.

All tubes, except the counting standards, were centrifuged at 1300 x g for 30 min at 4°C. The supernatant was discarded and 2.5ml of scintillation fluid were added to each tube including the counting standards. The tubes were capped and vortex mixed, and were then placed in a Canberra Packard Tricarb Series 4000 liquid scintillation counter to measure the amount of bound radioactivity in each sample. Counting of each tube was carried out for 4 min in the case of the $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ assays and for 10 min in the case of the PGE_2 assay. The methods used in the assay are summarised in Figure 7. The % tracer bound was calculated from the formula:-

$$\%\text{bound} = \frac{\text{counts in tube} \times 100}{\text{average standard counts}}$$

The average standard counts is the average count from tubes 5-8

Superfusion samples

Dispense an appropriate volume of each sample into tubes

↓

Evaporate off ethyl acetate in a stream of air at 45°C

Culture medium samples

Thaw and dispense an appropriate volume of each sample into tubes

Add the appropriate diluent to make up to the same volume as the PG standards

↓

Add 50µl of the appropriate [^{3}H]-PG to each tube

↓

Add 50µl of the appropriate diluted PG antiserum to each tube except the counting standards

↓

Vortex mix and incubate tubes at room temperature for 1h (for $PGF_{2\alpha}$ assays) or for 2h (for PGE_2 and 6-keto-$PGF_{1\alpha}$ assays)

↓

Add 50µl of NRS (not PGE_2 assays) and 50µl of DARS to each tube except the counting standards

↓

Vortex mix and incubate tubes at 4°C overnight

↓

Centrifuge tubes at 1300 x g for 30 mins (except counting standards)

↓

Discard supernatant

↓

Add 2.5ml of scintillation fluid and cap each tube

↓

Vortex mix tubes to resuspend the pellet

↓

Count each tube in a liquid scintillation counter for 4 min ($PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ assays) or for 10 min (PGE_2 assays)

Fig. 7. The general protocol for measurement of prostaglandins by radioimmunoassay

(the counting standards). Data from the counter were passed directly into an IBM-personal Computer programmed with the Packard Data Acquisition and Analysis (PC-DAAS). The Spline Curve Fit Programme was used which processes the standards according to a Modified Smooth Spline Algorithm. This programme subtracted the amount of non-specific binding from all standards then calculated the co-ordinates for the curve of best-fit. Using this standard curve, the computer calculated the amount of PG in each sample tube.

vi. Intra-assay coefficient of variation

This is a calculation of the variation between each sample duplicate.

Intra-assay coefficient of variation =

$$\frac{\text{standard deviation of sample duplicate} \times 100}{\text{mean value of sample duplicate}}$$

This coefficient was calculated by the computer for each unknown sample assayed in duplicate. If the coefficient for the sample was greater than 10%, the sample was re-assayed.

vii. Inter-assay coefficient of variation

This is a calculation of the variation between assays and is calculated using the average value obtained for the last two duplicates in the assay containing 0.32ng/ml of PG standard from several assays.

Inter-assay coefficient of variation =

$$\frac{\text{standard deviation of PG standard} \times 100}{\text{mean of PG standard}}$$

viii. Standard curves and estimates of precision for each assay

a. $PGF_{2\alpha}$

The standard curve obtained by averaging 5 consecutive $PGF_{2\alpha}$ assays is shown in Figure 8 (mean ± s.e.m.). The detection limit of the assay, defined as the amount of standard PG required to give a 10% fall in binding from the zero standard, was 8.5pg. The gradient of the linear portion of the standard curve for $PGF_{2\alpha}$ was 11.4% measured over a doubling of the concentration of $PGF_{2\alpha}$ per tube. If the gradient is less than 8% small differences in $PGF_{2\alpha}$ concentrations in samples produce changes in binding of tracer that are too small to be measured. From the 5 assays the inter-assay co-efficient of variation for the $PGF_{2\alpha}$ assay was 11%. The mean intra-assay coefficient of variation was 5.07%.

b. 6-keto-$PGF_{1\alpha}$

The standard curve obtained by averaging 8 consecutive 6-keto-$PGF_{1\alpha}$ assays is shown in Figure 9 (mean ± s.e.m.). The detection limit was 32pg. The gradient of the linear portion of the standard curve for 6-keto-$PGF_{1\alpha}$ was 12.2% measured over a doubling of the concentration of 6-keto-$PGF_{1\alpha}$ per tube. The inter-assay co-efficient of variation for the 6-keto-$PGF_{1\alpha}$ assay was 9.3%. The mean intra-assay co-efficient of variation was 5.48%.

c. PGE_2

The standard curve obtained by averaging 6 consecutive PGE_2 assays is shown in Figure 10 (mean ± s.e.m.). The detection limit of the assay was 65pg. The gradient of the linear portion of the standard curve for PGE_2 was 12.1% measured over a doubling of the concentration of PGE_2 per tube. The inter-assay co-efficient of

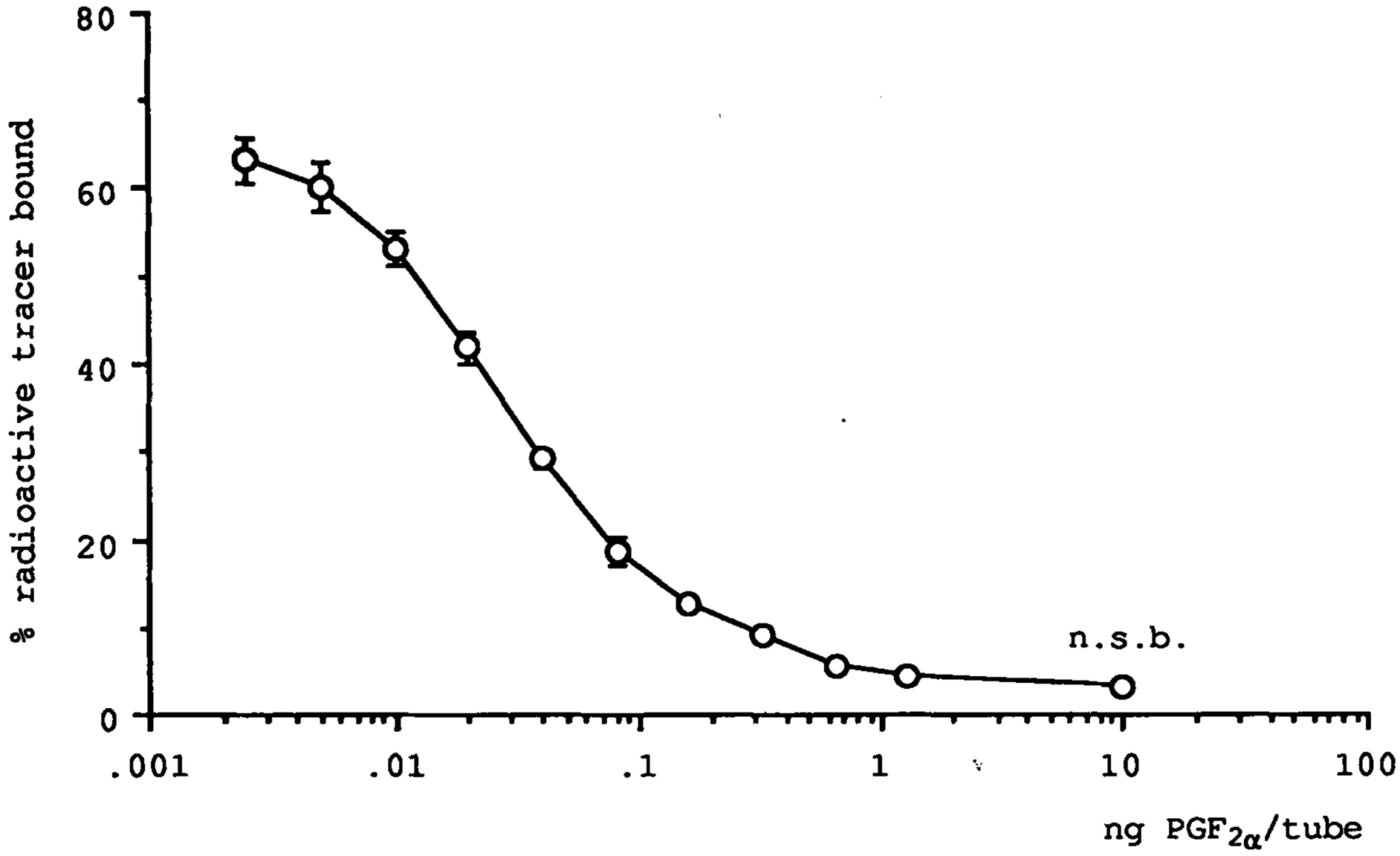


Fig. 8. Standard curve for $PGF_{2\alpha}$ radioimmunoassay. Each point is the mean ± s.e.m. (n = 5) although, in some instances, the size of the point is greater than the standard error (n.s.b.= non-specific binding).

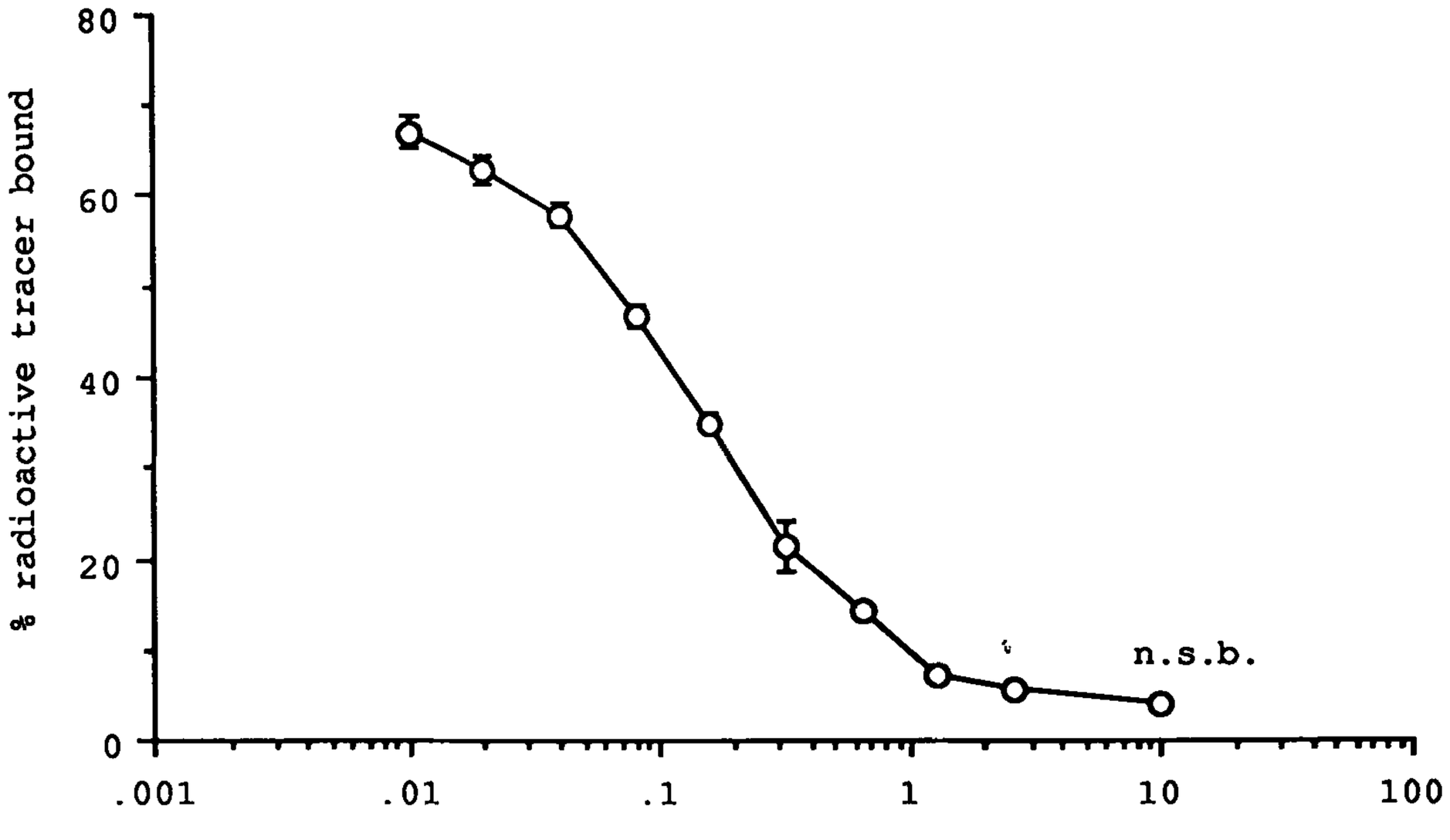


Fig. 9. Standard curve for 6-keto-PGF$_{1a}$ radioimmunoassay. Each point is the mean ± s.e.m. (n = 8) although, in some instances, the size of the point is greater than the standard error (n.s.b. = non-specific binding).

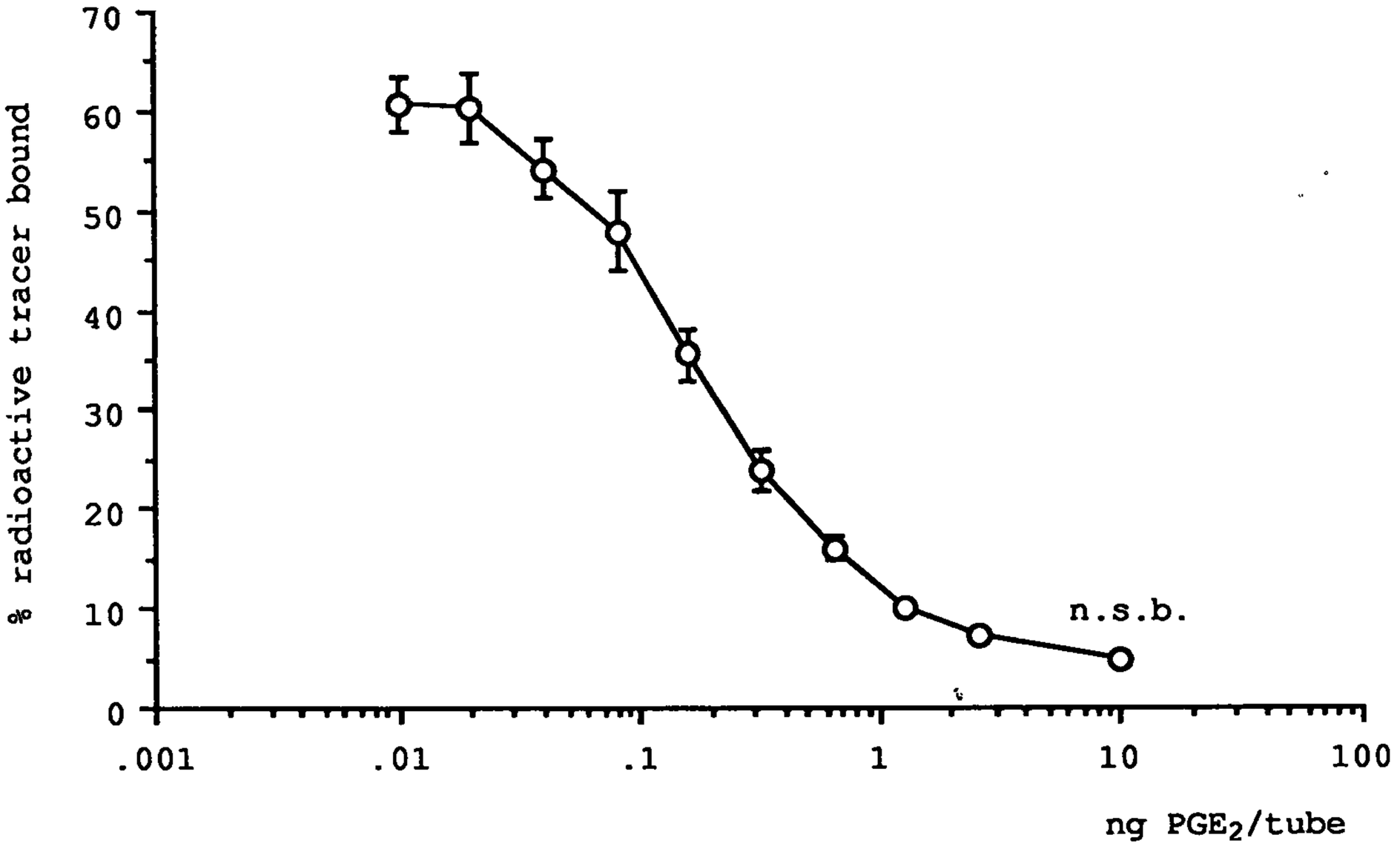


Fig. 10. Standard curve for PGE_2 radioimmunoassay. Each point is the mean ± s.e.m. (n = 6) although, in some instances, the size of the point is greater than the standard error (n.s.b.= non-specific binding).

variation for the PGE_2 assay--was 9.9%. The mean intra-assay co-efficient of variation was 5.34%.

2:10 Protein Purification and Separation

Introduction

Proteins released from guinea-pig endometrium and conceptus in culture were purified and separated by the various chromatographic techniques described in this section. The first step involved the removal of salts and other low molecular weight substances from the protein samples.

Method

All protein purification and separation procedures were carried out in the cold room at 4°C to prevent denaturation of the proteins by any proteases which might be present. The first step in the purification of proteins secreted from cultured endometrium or conceptus from Day-15 guinea-pigs was dialysis, which removed low molecular weight molecules present in the culture medium. The medium from each animal was placed in dialysis tubing which was tied at each end and dialysed against 5l of Tris buffer (2mM) adjusted to pH 8.2 with M HCl. This procedure was repeated 3 times. The dialysed medium was freeze-dried (lyophylized) and subjected to a desalting step on PD-10 disposable, desalting columns. The columns contain 9.1ml of Sephadex G-25 M and were equilibrated with 25ml distilled water. The lyophylized sample was dissolved in 2.5ml distilled water and allowed to run into the column. The protein was eluted in 3.5ml with distilled water and relyophylized before weighing. After dialysis and desalting, proteins were separated by a combination of chromatographic techniques. These were affinity chromatography on

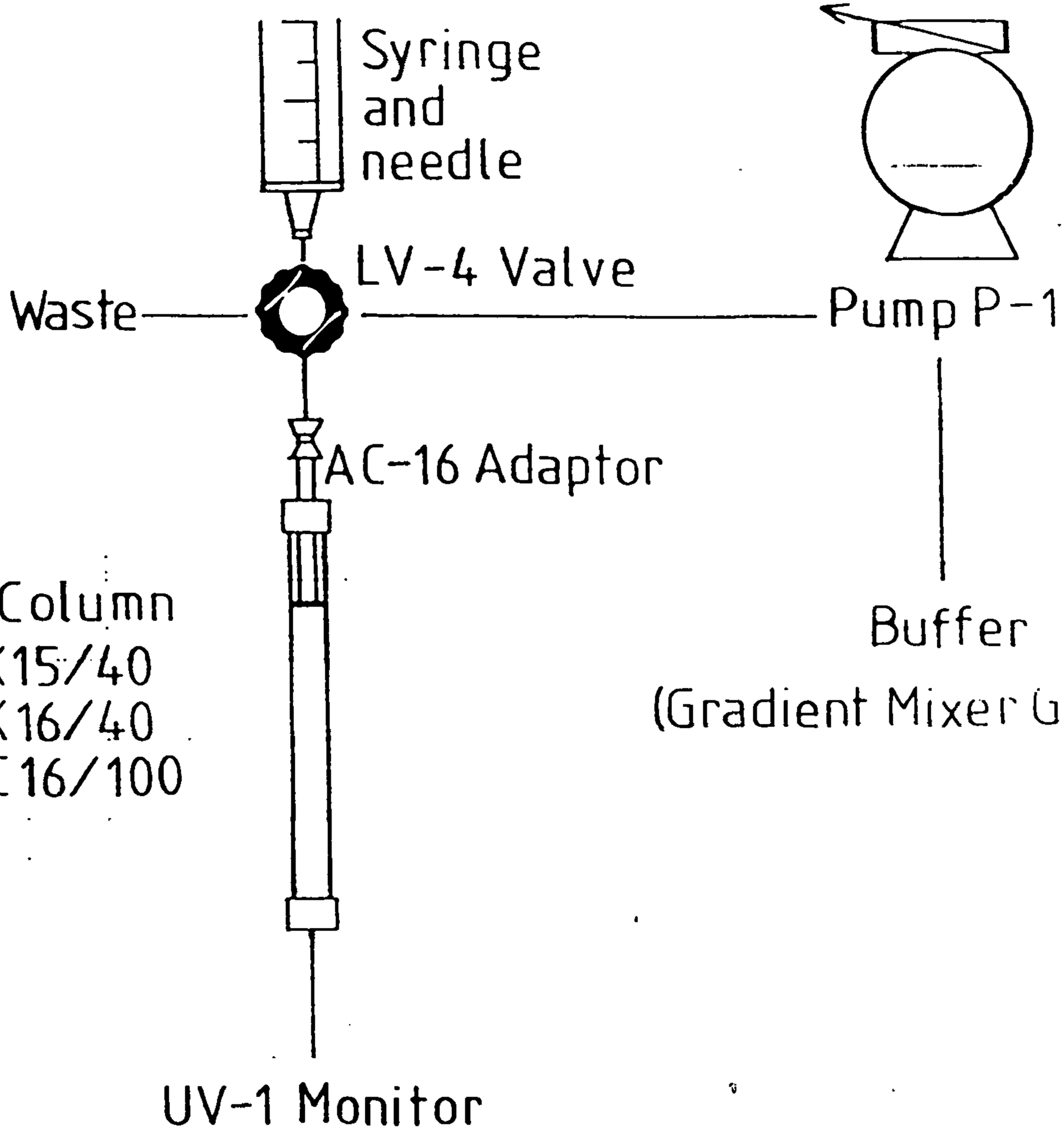


Fig. 11. The above diagram illustrates the arrangement of the equipment used for the purification of proteins, obtained from the guinea-pig endometrium and conceptus in culture, by affinity, ion-exchange and gel filtration chromatography.

Blue Sepharose CL-6B, ion-exchange chromatography on DEAE Sepharose CL-6B, and gel filtration chromatography on Sephadex G-75 Superfine (SF). The arrangement of the chromatography equipment used is shown in Figure 11.

To determine the amount of protein present in each dialysed, desalted and lyophylized sample a "Lowry assay" was performed. This method, described by Lowry, Rosebrough, Farr and Randall (1951), is based on the ability of protein molecules to combine with tartrate ions and Folin's phenolic reagent to form a complex which has a spectrophotometric absorbance at 750nm. The optical absorbance of this complex is linearly proportional to the total amount of proteins present in the solution.

Stock solutions were prepared as shown below

Solution A	1% (w/v) $CuSO_4.5H_2O$
Solution B	2% (w/v) NaK tartrate
Solution C	2% (w/v) Na_2CO_3 dissolved in 0.1M NaOH
Solution D	Folin's phenolic reagent diluted 1:1.5 with distilled water

Bovine serum albumin (BSA) 2.5 mg/ml

6 "standard" BSA solutions of known concentration were made up by diluting 0, 0.1, 0.2, 0.3, 0.4 and 0.5ml of BSA stock solution in separate test-tubes to 5ml with distilled water. Thus the 0.3ml of each "standard" protein solution used in each assay contained 0, 15, 30, 45, 60 and 75μg respectively. 0.5ml of Solution A was added to 0.5ml of Solution B, and to this mixture 50ml of Solution C were added. 3ml of this A+B+C solution were added to 0.3ml of each of the 6 "standard" protein solutions, and the tubes were vortex mixed and allowed to incubate for at least 15 min. 0.3ml of Solution D was then added to each "standard" protein solution and the tubes

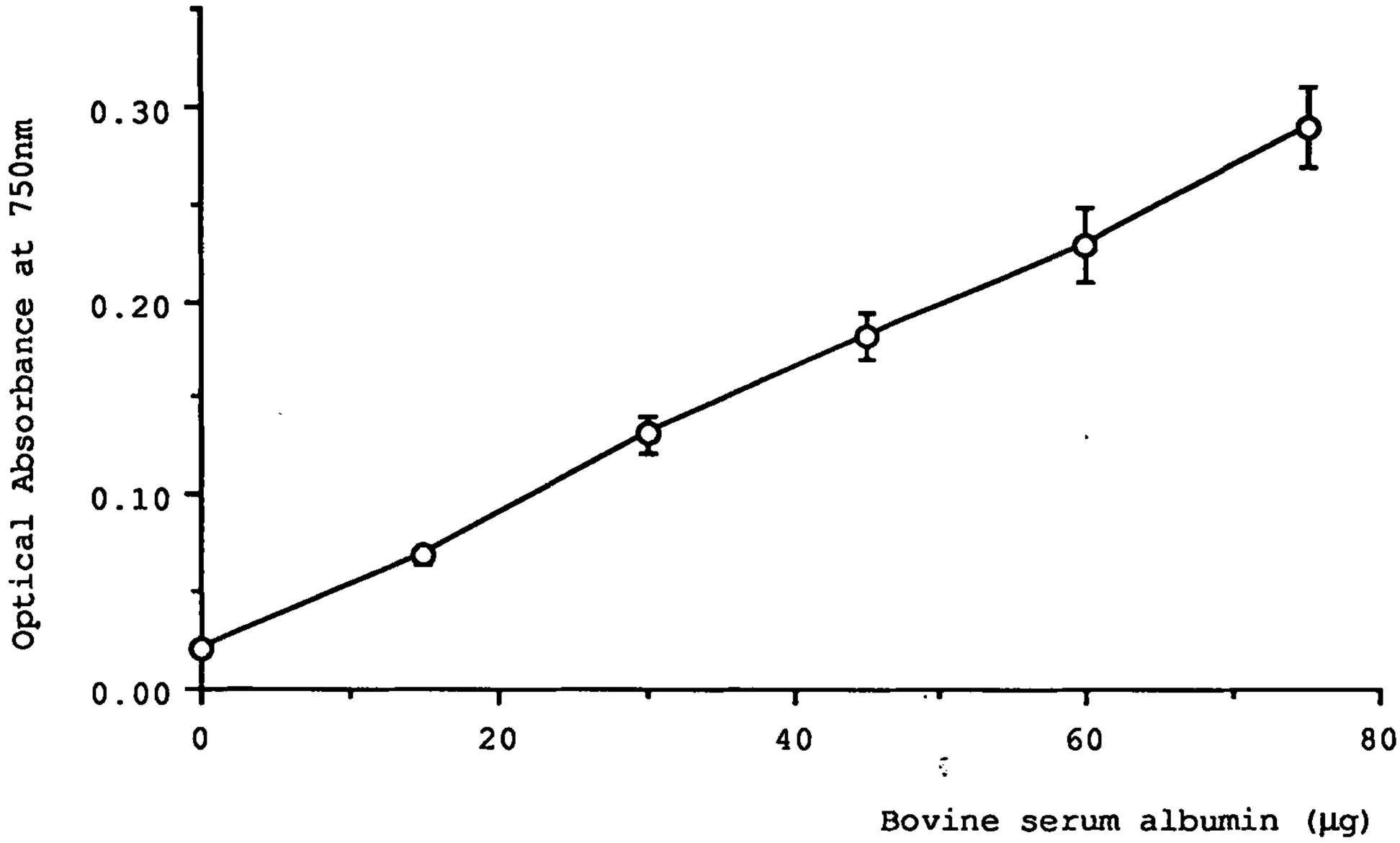


Fig. 12. Standard curve for Lowry protein assay. Each point is the mean ± s.e.m. ($n = 7$) although, in some instances, the size of the point is greater than the standard error.

were vortex mixed once again before incubating for a further 30 min. The Optical Absorbance of each protein standard at 750nm was measured in a Cecil^{TM} Spectrophotometer. The absorbance data obtained were used to construct a plot of optical absorbance against the amount of protein present. Samples to be measured for protein content were diluted to 0.3ml with distilled water and were subjected to the same procedure as described above for the "standard" proteins. Absorbance values obtained from these samples were applied to the standard plot and the protein content calculated. A new standard plot was constructed for each assay in order to account for any variations in making up of solutions or duration of incubation times between assays. Figure 12 shows the regression line obtained when applied to the combined results (mean $\pm$ s.e.m.) of the standard curves from 7 consecutive protein assays. The line had a regression co-efficient of 0.99. It was concluded that the method of Lowry et al. was a reliable assay for measuring the concentration of proteins in samples.

i. Affinity Chromatography

Introduction

Affinity chromatography involves the molecule to be purified being specifically and reversibly adsorbed by a ligand coupled to the gel. Axen, Porath and Ernback (1967) first reported that biologically active proteins could be coupled to cellulose, starch and cross-linked polysaccharides such as Sephadex to give a high protein yield. Blue Sepharose CL-6B consists of the dye Cibacron Blue F3 G-A covalently attached to the cross-linked agarose gel Sepharose CL-6B. The concentration of coupled dye is approximately 2μmol/ml of swollen gel. Cibacron Blue F3 G-A binds a wide variety

of enzymes and other proteins, including enzymes requiring adenylyl-containing co-factors (including NAD^+ and $NADP^+$) (Thompson, Cass and Stellwagen, 1975), coagulation factors (Swart and Henker, 1970), interferon (Jankowski, Von Muenchhausen, Sulkowski and Carter, 1976) and albumin (Travis, Bowen, Tewksbury, Johnson and Pannell, 1976). Albumin and interferon bind in a less specific manner than proteins which require nucleotide cofactors. Less specifically bound proteins require high concentrations of salt for elution.

Human uterine flushings have been shown to be highly contaminated with serum proteins particularly albumin (Edwards, Talbert, Israelstam, Nino and Johnson, 1968; Roberts, Parker and Henderson, 1976a) which comprises 30-50% of total serum protein. Proteins from human uterine flushings were successfully depleted of albumin by affinity chromatography on Blue Sepharose CL-6B (MacLaughlin and Richardson, 1983). As preliminary polyacrylamide gel electrophoresis (PAGE) results for endometrium and conceptus secretory proteins showed a heavily stained band corresponding to the molecular weight marker for albumin, it was decided to subject all protein samples to affinity chromatography on Blue Sepharose CL-6B. Both unbound proteins which passed through the column and those proteins which were retained on Blue Sepharose were collected for further separation. This was particularly important as Blue Sepharose binds interferon with which the antiluteolytic proteins secreted by the embryos of sheep (Godkin et al., 1984a) and cows (Helmer et al., 1989a) have been shown to have close homology (Stewart et al., 1987; Imakawa et al., 1988).

Method

a. Swelling and washing the gel

Freeze-dried Blue Sepharose CL-6B powder (10g) was washed with 2l distilled water in a sintered glass funnel for 15 min. The swollen gel was equilibrated in Affinity Running Buffer consisting of 50mM Tris adjusted to pH 8.0 with HCl and containing 0.1M KCl. The gel suspension was degassed and allowed to cool to 4°C in the cold room.

b. Filling and packing the column

The gel was allowed to settle and excess buffer decanted to leave a 75% gel solution. Decantation also removes any fine particles present. Affinity Running Buffer was injected into the outlet tubing of a Pharmacia K15/40 column until it passed through the bed support in order to displace all air from the dead space. The outlet tubing was closed and the mixed gel suspension was poured carefully down the inside wall of the tilted column. Once all the gel was poured, the column was filled to the top with Affinity Running Buffer, the column top piece was screwed on, and any air removed via the air vent in the top piece. Immediately after filling the column, the pump was started and the column was packed at a rate of 0.7ml/min until a stable bed height of approximately 20cm was obtained. 2-3 column volumes of Affinity Running Buffer were passed through the column in order to stabilise the bed.

c. Application of sample to the column

Lyophylized protein was dissolved in Sample Buffer which consisted of Affinity Running Buffer containing 5% (v/v) glycerol. The glycerol increased the density of the protein sample allowing

it to flow into the column with less mixing. The column top piece was removed and the buffer above the gel bed was sucked out. The sample was applied to the bed with great care to minimise disturbance of the gel. This was achieved by using a syringe attached to a piece of fine capillary tubing and running the sample down the inside of the column wall from about 1cm above the bed. The column outlet was opened long enough to allow the sample to run into the gel. The column was refilled with Affinity Running Buffer, the top piece replaced and air removed via the air vent. The pump was started and Affinity Running Buffer allowed to run through the column at the rate of 0.1ml/min. The passage of proteins from the column was monitored by measuring the absorbance of the effluent at 280nm on a UV-1 monitor connected to a Rec-481 chart recorder. Non-bound proteins were collected in a Frac-100 fraction collector and enough Affinity Running Buffer was passed through the column to ensure that all unbound proteins were eluted.

d. Elution of bound proteins

The Affinity Running Buffer above the gel was replaced with Elution Buffer consisting of 50mM Tris adjusted to pH 8.0 with HCl and containing 1.5M KCl. This high salt buffer was pumped through the column at 0.1ml/min to elute all bound proteins. Collected unbound and bound proteins were dialysed, desalted and lyophylized before weighing.

e. Regeneration of Blue Sepharose CL-6B

The gel was regenerated after each run by removing from the column and washing in a sintered glass funnel with 4-5 washing cycles of alternate:-

1. 2 x Vt (Vt=total bed volume = 100ml) of 0.1M Tris-HCl, pH 8.5, 0.5M NaCl.
2. 2 x Vt (100ml) of 0.1M sodium acetate, pH 4.5, 0.5M NaCl.

The column was then re-equilibrated with Affinity Running Buffer. The gel was degassed and allowed to cool to 4°C in the cold room before repacking.

ii. Ion-exchange Chromatography

Introduction

Separation of molecules by ion-exchange chromatography is achieved on the basis of the charge carried by each molecule. Molecules are reversibly adsorbed onto the ion-exchange resin and eluted from the column by varying either the ionic strength or the pH of the buffer, thus changing the affinity of the molecule for the ion-exchanger. The ion-exchanger consists of an insoluble matrix to which charged groups are covalently bound. The weakly basic anion exchanger diethylaminoethyl (DEAE) was used in the following procedures. Weak ion exchangers have a degree of dissociation and thus an exchange capacity which varies markedly with pH, whereas strong ion exchangers are ionized over a wide pH range. Anion exchangers are positively charged with negatively charged counter-ions. The DEAE was attached by ether linkages to the monosaccharide units on Sepharose CL-6B. Sepharose CL-6B has excellent flow properties and an extremely stable bed volume that is relatively insensitive to changes in ionic strength and pH. Sepharose CL-6B shows extremely low non-specific adsorption of molecules.

Method

a. Preparation of the gel

DEAE Sepharose CL-6B (50 ml), which was supplied preswollen, was equilibrated with DEAE Starting Buffer in a sintered glass funnel. The buffer consisted of 10mM Tris adjusted to pH 8.2 with HCl. The gel suspension was degassed and allowed to cool to 4°C in the cold room.

b. Filling and packing the column

The bottom piece was removed from a Pharmacia K16/40 column and replaced with a Pharmacia AC16 adaptor to shorten the column length. Buffer was injected into the outlet tubing to displace air from the dead space under the bed support. The outlet tubing was closed and the gel was carefully poured down the inside wall of the tilted column. The column was filled with buffer until it formed an upward meniscus, and an AC16 adaptor was inserted at an angle to avoid trapping air under the net. Adaptors allow automatic sample application which eliminates disturbance of the gel bed. The tightening mechanism on the adaptor was adjusted to form a seal on the column wall and the adaptor top piece was screwed onto the column top piece. The adaptor was slowly pushed down the column, allowing buffer to displace the air above the net and in the capillary tubing, until it rested just above the gel bed. The adaptor was locked in position, and the column was packed at a rate of 1.5ml/min until a stable bed height of 25cm was obtained.

c. Sample application

The protein sample was dissolved in enough Sample Buffer to give not more than a 30mg/ml solution. The Sample Buffer consisted of

DEAE Starting Buffer with 5% (v/v) glycerol. The sample was placed in a syringe (minus the plunger), attached to an LV-4 valve and allowed to run into the column under gravity.

d. Elution of proteins

The pump was started and 200ml buffer forming a continuous ionic gradient ranging from 0 to 0.5M NaCl, were run through the column at a rate of 0.4ml/min. This was achieved by using the Pharmacia Gradient Mixer GM-1. 100ml DEAE Starting Buffer (lacking NaCl) were placed in the mixing chamber and 100ml DEAE Starting Buffer containing 0.5M NaCl were placed in the other chamber. The mixing blade was switched on and, as buffer was removed from the mixing chamber by the pump, it was replaced by the 0.5M NaCl buffer from the other chamber via an interconnection between the chambers, so a continuous ionic gradient was formed. Eluted proteins were collected on the fraction collector, dialysed, desalted and lyophylized.

e. Regeneration of the gel

The column was re-equilibrated with 2-3 column volumes of DEAE Starting Buffer before each run to wash out the NaCl. The ion-exchanger was regenerated intermittently to remove strongly bound molecules by washing the bed with the following solutions:-

1. 1 x Vt (Vt=total bed volume = 50ml) sodium acetate (1M, adjusted to pH 3.0 with HCl)
2. 1.5 x Vt (75ml) sodium hydroxide (0.5M). This solution was left in the column overnight.
3. 1.5 x Vt (75ml) sodium acetate (1M, pH 3.0)

As Sepharose is a rigid and stable gel, it could be regenerated within the column without the need for repacking. The column was

re-equilibrated with DEAE Starting Buffer.

iii. Gel Filtration Chromatography

Introduction

Gel filtration chromatography separates proteins on the basis of the molecular weight. Proteins with low molecular weights diffuse into pores in the gel and thus are retained in the column for a longer time than proteins with large molecular weights which are excluded from the gel pores by size. Large proteins pass out of the column faster than small proteins, and protein molecules are therefore eluted in order of decreasing molecular size. The fractionation range of a gel is determined by the size of the gel pores. Sephadex G-75 Superfine (SF) was used in the following procedures to separate high molecular weight proteins (>70,000) from low molecular weight proteins. Sephadex is a bead-formed gel prepared by cross-linking dextran with epichlorohydrin. Sephadex G-75 SF has a dry bead diameter of 10-40μm and a fractionation range of 3,000-70,000. The Superfine grade gives high resolution but high back pressures mean that this is at the expense of flow rate.

Method

a. Preparation of the gel

Sephadex G-75 SF was supplied as a dried powder. 15g of the powder was swollen in 2.25l distilled water overnight. The swollen gel was equilibrated in Running Buffer consisting of 10mM Tris adjusted to pH 8.2 with HCl and containing 0.33M NaCl. The gel suspension was degassed and allowed to cool to 4°C. After settling, the smallest particles were removed by decantation. The decanting step was repeated 3 times as small particles can retard flow through the gel.

b. Filling and packing the column

Running Buffer was injected into the outlet tubing of a Pharmacia C16/100 column to displace any air from under the bed support. The outlet tubing was closed and a gel reservoir was attached to the column, before pouring the gel down the inside of the tilted column wall. After righting the column, the reservoir was filled to the top with Running Buffer and flow through the column was started. The gel was packed at a rate of 0.1ml/min (low flow rates are necessary with Sephadex G-75 SF as the gel may become compressed with high flow rates) until all the gel had packed into the column. The pump was stopped and the buffer was sucked out of the reservoir. The reservoir was removed and replaced with a Pharmacia AC16 adaptor.

The adaptor was inserted into the gel at an angle to avoid trapping air under the net and the tightening mechanism was adjusted to form a seal on the column wall. The adaptor was pushed down the column, displacing air from above the net and in the capillary tubing, until it rested just above the gel bed. The adaptor was locked into position and flow through the column was restarted at a rate of 0.1ml/min. Further packing was carried out until a stable bed height of 86cm was obtained. The adaptor was readjusted down to the surface of the gel bed.

c. Void volume determination

The void volume (Vo) of a column is the volume of buffer required to elute very large molecules, which are excluded from the pores of the gel by size, from the column. The void volume is therefore a measure of the mobile phase which is the total volume of the packed bed (Vt) minus the volume of the stationary phase (Vs) which comprises the gel forming substance plus the volume of the gel

pores.

The high molecular weight (>2,000,000) protein Blue Dextran was used to determine the void volume of the column. As the colour of this protein shows up against the gel, it is also useful for checking for any heterogeneities in the gel bed by watching its progress through the column. Blue Dextran (2mg) was dissolved in Sample Buffer which consisted of Running Buffer with 5% (v/v) glycerol. The sample was applied to the column under gravity via a syringe attached to an LV-4 valve and flow through the column was started at a rate of 0.1ml/min. The void volume was taken as the volume of buffer passed through the column from the point of Blue Dextran application to the centre of the peak of eluted Blue Dextran.

d. Calibration of the column

The molecular weights of the sample proteins were determined by comparing the ratio Ve/Vo for the sample protein with Ve/Vo for standard proteins (Table 7) of known molecular weight where Ve is the elution volume of the protein. The concentrations of standard proteins used were calculated by the manufacturer to give an A_{280} of approximately 1.0. The protein standards were applied to the column individually and run at a rate of 0.1ml/min. The elution volume (Ve) for each standard protein was determined, and Ve/Vo was calculated and was plotted versus the logarithm of the molecular weight for each standard protein. Figure 13 shows the calibration curve obtained for Sephadex G-75 SF using the Pharmacia MW-GF-70 calibration kit.

Table 7. The source, molecular weight and concentration of each protein from the Pharmacia MW-GF-70 Calibration Kit used to calibrate the Sephadex G-75 SF column. Each protein was made up in 2ml of Sample Buffer.

Protein	Source	M.W.	Concn (mg/ml)
Albumin	Bovine serum	66,000	5
Carbonic anhydrase	Bovine erythrocyte	29,000	2
Cytochrome c	Horse heart	12,400	2
Aprotinin	Bovine lung	6,500	3

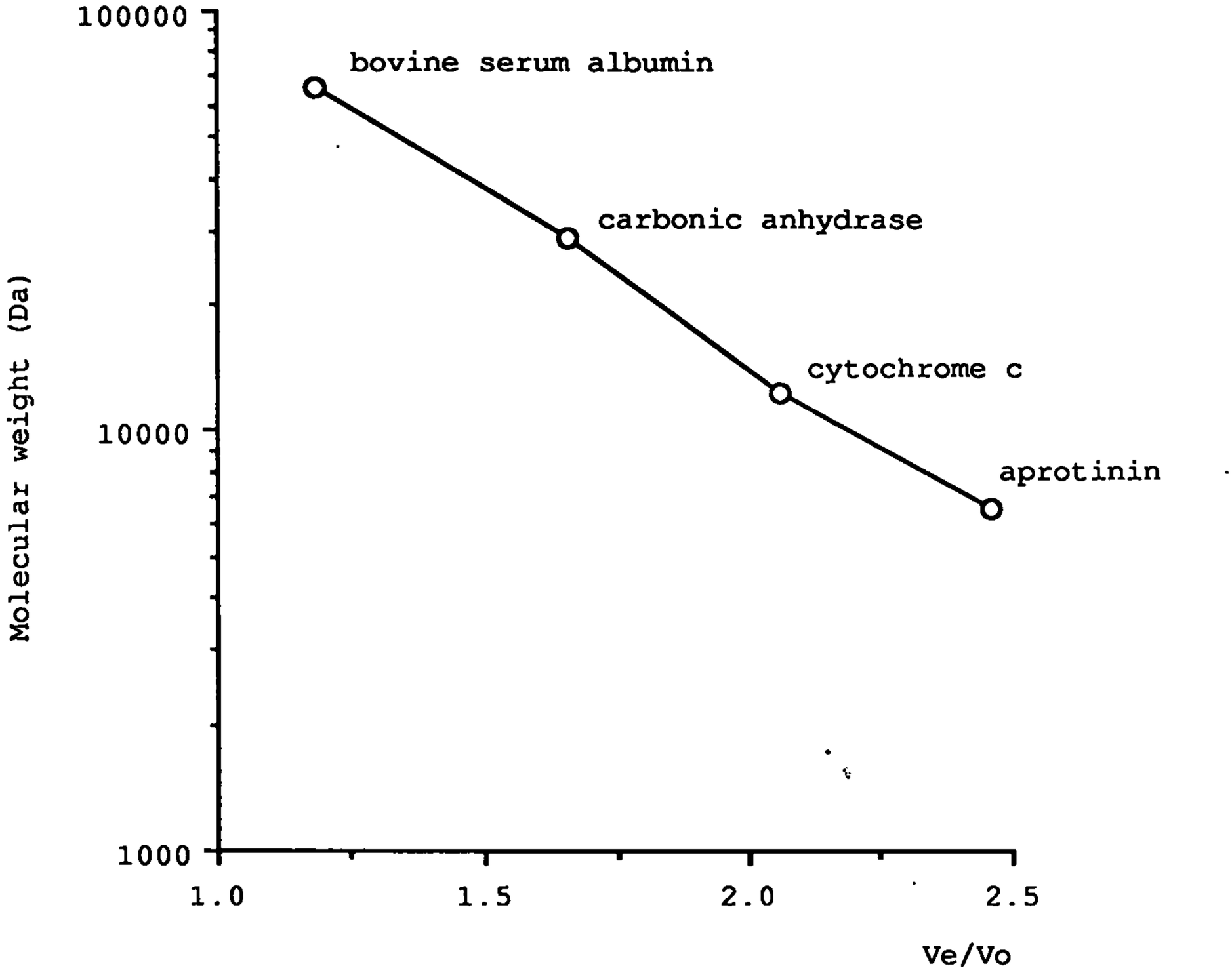


Fig. 13. Calibration curve obtained with proteins from the MW-GF-70 Kit run on Sephadex G-75 SF. Vo = the void volume (i.e. the elution volume of Blue Dextran) and Ve = the elution volume of the protein standard.

e. Molecular weight determination for sample proteins

Sample proteins were dissolved in 2ml of Sample Buffer, applied to the column and run at 0.1ml/min. The elution volume for each separated protein was determined and Ve/Vo was calculated. The molecular weight was obtained from the standard curve (Figure 13). Separated proteins were dialysed, desalted and lyophylized.

2:11 Overall conclusions

The general methods described here have been developed and tested in order to:

1. Culture guinea-pig endometrium and conceptus tissue for periods up to 24h.
2. Allow the superfusion of the guinea-pig uterus *in vitro*.
3. Assay the amounts of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ released by the uterus and endometrium during superfusion and culture respectively.
4. Monitor protein synthesis by endometrial tissue *in vitro*.
5. Isolate and purify proteins released by guinea-pig endometrium and conceptus during culture.

It was concluded that the methods had been developed and assessed to a sufficiently high standard to allow meaningful results to be obtained. The next sections describe the experiments performed with these general methods based on the aims of the overall investigation stated in the General Introduction.

SECTION 3

RESULTS

3:1 INVESTIGATIONS INTO THE INVOLVEMENT OF G-PROTEINS IN THE REGULATION OF PROSTAGLANDIN RELEASE FROM THE GUINEA-PIG ENDOMETRIUM

3:1:a The effects of cholera toxin, pertussis toxin and sodium fluoride on the output of prostaglandins from guinea-pig endometrium in culture

Introduction

The release of $PGF_{2\alpha}$ from the uterus is responsible for luteal regression at the end of the cycle in several non-primate species including the guinea-pig (see Horton and Poyser, 1976). $PGF_{2\alpha}$ output from the guinea-pig uterus superfused *in vitro* increased 21.9-fold between Day 7 and Day 15 of the oestrous cycle (Poyser and Brydon, 1983) and, as $PGF_{2\alpha}$ is not stored in significant amounts in the guinea-pig uterus (Poyser, 1972), the increase in $PGF_{2\alpha}$ release towards the end of the cycle must be due to a stimulation of $PGF_{2\alpha}$ synthesis from arachidonic acid. As bound arachidonic acid cannot be converted to PGs (Lands and Samuelsson, 1968; Vonkeman and Van Dorp, 1968) and the concentration of free arachidonic acid in the guinea-pig uterus is very low (Leaver and Poyser, 1981), the release of arachidonic acid from some bound source must precede the synthesis of $PGF_{2\alpha}$. Of the total arachidonic acid in the guinea-pig uterus, 93% was esterified to phospholipids, of which 80% was bound to phosphatidylcholine (PC) and phosphatidylethanolamine (PE). There is a significant decrease in the quantity of arachidonic acid bound to PC between Days 7 and 15 of the oestrous cycle (Leaver and Poyser, 1981). Further studies on arachidonic acid turnover in

guinea-pig endometrium revealed that PC and PE, but not phosphatidylinositol (PI), are the probable source of arachidonic acid for increased endometrial $PGF_{2\alpha}$ synthesis at the end of the cycle (Ning et al., 1983; Ning and Poyser, 1984). This implies that, in the guinea-pig uterus, the activation of phospholipase (PL) A_2 is responsible for PG formation. The activity of microsomal PLA_2 from guinea-pig endometrial homogenates increased 1.5-fold between Day 7 and Day 16 of the cycle (Downing and Poyser, 1983), and thus regulation of the activity of PLA_2 is a likely site for the control of $PGF_{2\alpha}$ production.

PLA_2 is activated by a pertussis-toxin sensitive G-protein in guinea-pig neutrophils (Bokoch and Gilman, 1984), rat thyroid cells (Burch, Luini and Axelrod, 1986), rod outer segments of bovine retina (Jelsema and Axelrod, 1987) and fibroblasts (Burch and Axelrod, 1987). Also, PGI_2 synthesis by bovine aortic endothelial cells (Pirotton, Erneux and Boeynaems, 1987) and PGE_2 synthesis by a murine macrophage cell line (Burch, Jelsema and Axelrod, 1988) are enhanced by pertussis toxin and cholera toxin, and fluoride stimulates PGI_2 release from the rat aorta (Jeremy and Dandona, 1988). As the increase in uterine $PGF_{2\alpha}$ production from the guinea-pig uterus at the end of the cycle is mediated via an increase in protein synthesis (Poyser, 1979; Poyser and Riley, 1987; Riley and Poyser, 1989), it may be that oestradiol acting on a progesterone-primed uterus stimulates the formation of and/or causes the activation of a G-protein in guinea-pig endometrium which switches on $PGF_{2\alpha}$ synthesis by activating PLA_2. Therefore, the effects of the G-protein modulators cholera toxin, pertussis toxin and sodium fluoride on the production of PGs by guinea-pig endometrium in culture have been investigated.

Methods

The uteri were removed from five Day-7 and five Day-15 guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 14 petri-dishes containing 15-30mg wet weight (3-6mg dry weight) of endometrium were prepared from each uterus. Pairs of dishes were treated with 10ng/ml cholera toxin, 100ng/ml cholera toxin, 10ng/ml pertussis toxin, 100ng/ml pertussis toxin, 10mM sodium fluoride, 0.16mM dithiothreitol (reagent control) and no treatment (control). The cholera toxin and pertussis toxin were incubated for 30 min at 37°C with dithiothreitol (12.5mM) to reduce the sulphide groups, allowing activation of ADP-ribosyl-transferase, prior to addition to the dishes. Consequently, the dishes containing cholera toxin and pertussis toxin also contained 0.16mM dithiothreitol. The petri dishes were incubated at 37°C for 24h, and the culture medium was removed and replaced with fresh medium containing the same treatments every 8h. The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each petri dish into preweighed containers and dried by placing in an oven at 37°C for 24 hours. Each container was then reweighed and the amount of dried endometrium from each petri dish was calculated. The outputs of PGs were calculated per mg dry weight of endometrium.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's

multiple range test or, if the variances of the groups were significantly different by the variance ratio F test, by a modified t test for unequal variances. Differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

Change in PG outputs with time

The control outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium and of 6-keto-$PGF_{1\alpha}$ from Day-7 endometrium significantly ($P < 0.05$) declined during 24h of culture (Figures 14 and 15). The control output of $PGF_{2\alpha}$ from Day-7 endometrium significantly ($P < 0.05$) increased while the control output of PGE_2 from the same tissue did not significantly change during 24h of culture (Figures 14 and 15).

Effects of treatments

Dithiothreitol (0.16mM) had no significant effect on endometrial output of any of the 3 PGs measured during any of the 3 successive 8h periods of culture, indicating that dithiothreitol alone does not interfere with PG synthesis (Figures 14 and 15).

Cholera toxin and pertussis toxin (10 and 100ng/ml) had no significant effects on the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 and Day-15 guinea-pig endometrium in culture for 24h (Figures 14 and 15).

Sodium fluoride (10mM) tended to increase the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 endometrium after 8h of culture and this stimulation was significant ($P < 0.05$) for 6-keto-$PGF_{1\alpha}$

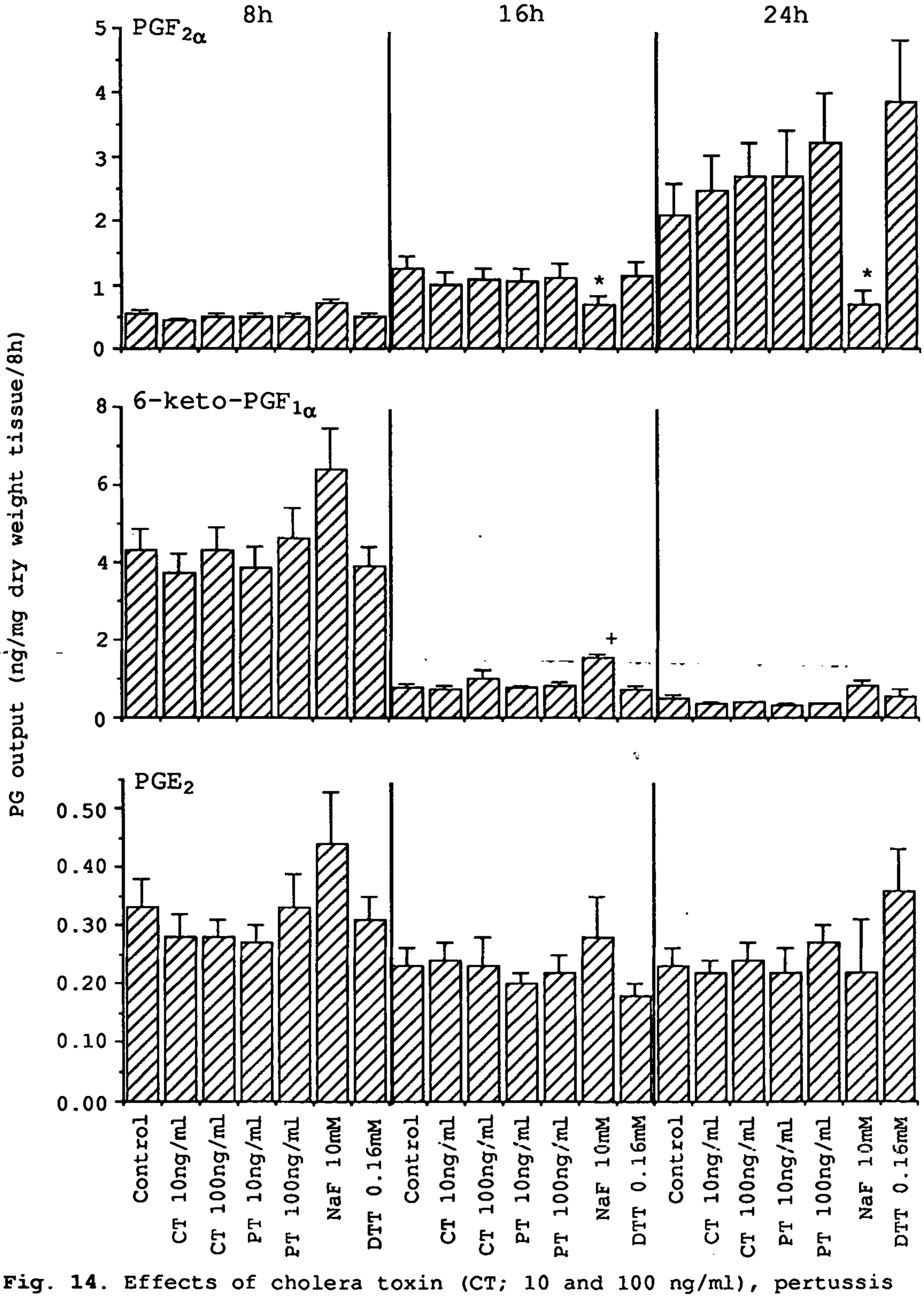


Fig. 14. Effects of cholera toxin (CT; 10 and 100 ng/ml), pertussis toxin (PT; 10 and 100 ng/ml), sodium fluoride (NaF; 10mM) and dithoithreitol (DTT; 0.16mM) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium cultured for 24h and sampled every 8h. *Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

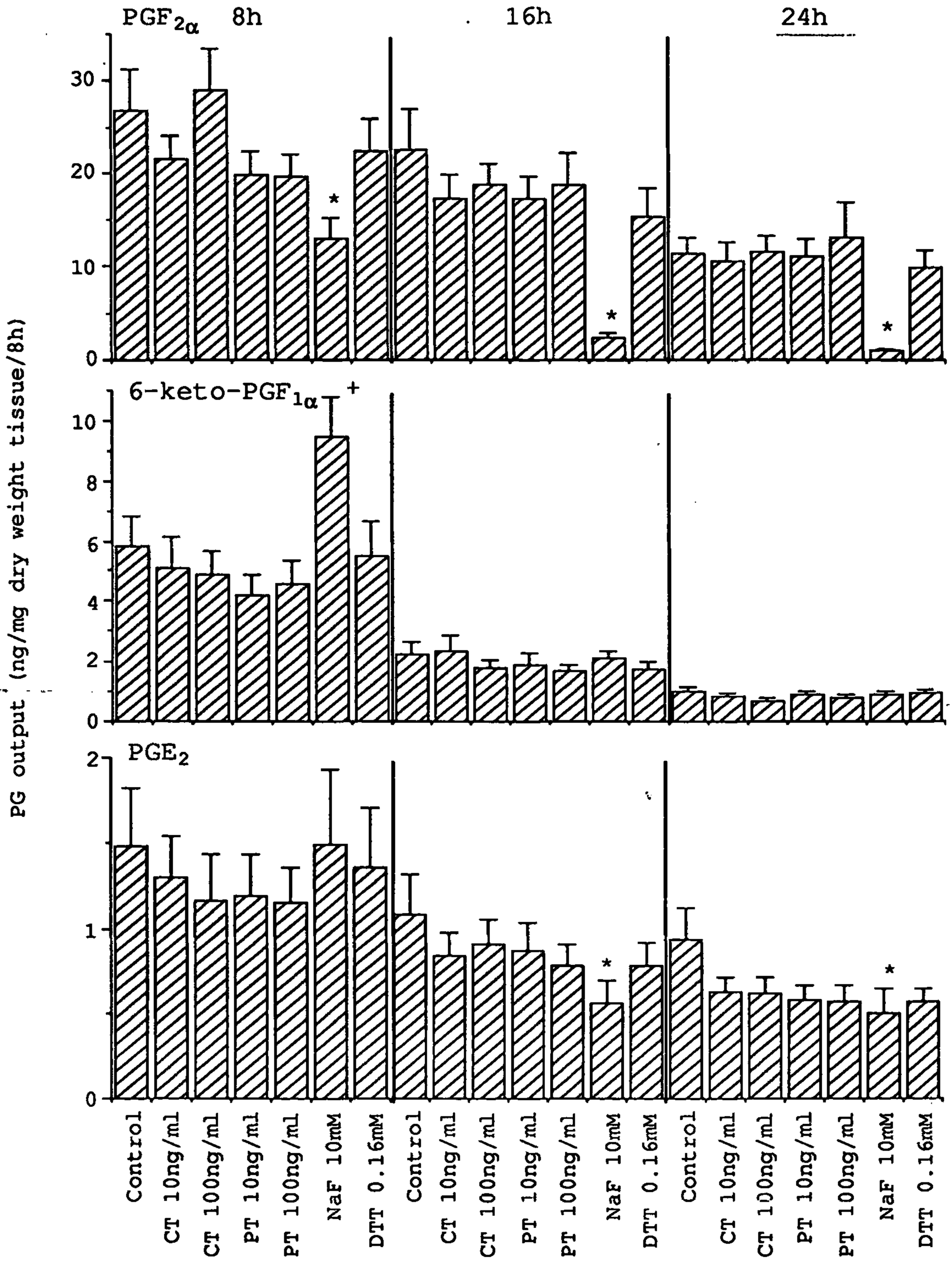


Fig. 15. Effects of cholera toxin (CT; 10 and 100 ng/ml), pertussis toxin (PT; 10 and 100 ng/ml), sodium fluoride (NaF; 10mM) and dithiothreitol (DTT; 0.16mM) on mean (± s.e.m., $n = 10$) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium cultured for 24h and sampled every 8h.*Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time.+Significantly higher ($P < 0.05$) than the corresponding control value for the same PG at the same time.

after 16h of culture (Figure 14). However, 10mM sodium fluoride significantly ($\underline{P} < 0.05$) inhibited $PGF_{2\alpha}$ output from Day-7 endometrium after 16 and 24h of culture (Figure 14).

Sodium fluoride (10mM) significantly ($\underline{P} < 0.05$) inhibited $PGF_{2\alpha}$ output after 8h, and $PGF_{2\alpha}$ and PGE_2 outputs after 16 and 24h of culture from Day-15 endometrium (Figure 15). The output of 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium was significantly ($\underline{P} < 0.05$) increased by sodium fluoride after 8h of culture (Figure 15).

Conclusions

Comparison of Figures 14 and 15 reveals that the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ during the first 8h period of culture are 48.7-, 4.5- and 1.3-fold higher from Day-15 endometrium than from Day-7 endometrium, respectively. Therefore the output of $PGF_{2\alpha}$ from guinea-pig endometrium is specifically stimulated between Day-7 and Day-15 of the cycle. Cholera toxin and pertussis toxin, in concentrations which normally ADP-ribosylate and influence G-protein activity, had no effect on the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 and Day-15 guinea-pig endometrium. It therefore appears that toxin-sensitive G-proteins are not involved in the processes which control $PGF_{2\alpha}$ synthesis in the guinea-pig endometrium.

Sodium fluoride (10mM) had two opposite effects on endometrial PG production. In the short term, sodium fluoride stimulated endometrial PG output but, in the longer term, an inhibitory effect prevailed. The mechanism by which sodium fluoride inhibits PG production from guinea-pig endometrium in culture was investigated in the next section (3:1:b). The stimulatory effect was evident but was not significant for any of three PGs during the first 8h period

of culturing Day-7 endometrium. However, the stimulation was significant for 6-keto-$PGF_{1\alpha}$ output from Day-7 endometrium at 16h and from Day-15 endometrium at 8h. Sodium fluoride (10mM) inhibited the outputs of $PGF_{2\alpha}$ from Day-7 and Day-15 endometrium, and the output of PGE_2 from Day-15 endometrium, without inhibiting the output of 6-keto-$PGF_{1\alpha}$ on either day. These results suggest that 6-keto-$PGF_{1\alpha}$ output is more sensitive to stimulation by sodium fluoride, as the stimulatory effect of sodium fluoride lasted longer for 6-keto-$PGF_{1\alpha}$ than for $PGF_{2\alpha}$ and PGE_2. Alternatively, the inhibitory effect of sodium fluoride may have been greater for $PGF_{2\alpha}$ and PGE_2 than for 6-keto-$PGF_{1\alpha}$. The fact that sodium fluoride initially stimulated endometrial PG synthesis, whereas cholera and pertussis toxins had no effect, raises the possibility that PG synthesis may be controlled by a G-protein which is fluoride sensitive and toxin-insensitive.

3:1:b. The effect of sodium fluoride on the incorporation of [^{3}H]-leucine into cellular and secreted proteins by Day-15 guinea-pig endometrium in culture

Introduction

The presence of actinomycin D, cycloheximide or puromycin in the culture medium inhibited the outputs of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 from Day-7 and Day-15 guinea-pig endometrium in culture (Riley and Poyser, 1989). Actinomycin D, cycloheximide and puromycin also inhibited the synthesis of secreted and cellular proteins by Day-7 and Day-15 endometrium in culture (Riley and Poyser, 1989), implying that endometrial synthesis of PGs is dependent upon endometrial protein synthesis.

As the previous experiment (see Section 3:1:a) showed that sodium fluoride (10mM) inhibited PG synthesis, and particularly $PGF_{2\alpha}$ synthesis, by guinea-pig endometrium in culture, it was decided to investigate whether sodium fluoride was acting via a similar mechanism as protein synthesis inhibitors. Therefore, in the following experiment the effect of sodium fluoride on the incorporation of [^{3}H]-leucine into both cellular and secreted proteins of Day-15 guinea-pig endometrium maintained in culture was examined. The incorporation of [^{3}H]-leucine into proteins gives a measure of de novo protein synthesis.

Methods

The uteri were removed from five Day-15 guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 6 petri dishes

containing 25-30mg wet weight of endometrium were prepared from each uterus. Each dish contained 10μCi [^{3}H]-leucine and pairs of dishes were treated with 2mM sodium fluoride, 10mM sodium fluoride and no treatment (control). The endometrium was cultured at 37°C for 24h, and the amounts of [^{3}H]-leucine incorporated into endometrial secreted and cellular proteins were measured by the methods described in Section 2:6. The incorporation of [^{3}H]-leucine into cellular and secreted proteins was calculated per mg wet weight of endometrium. The results for the nonspecific binding of [^{3}H]-leucine to cellular and secreted proteins were subtracted from the respective results for [^{3}H]-leucine incorporation into cellular and secreted proteins.

Statistical tests

Differences between treated and control groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

10mM sodium fluoride, but not 2mM sodium fluoride, significantly ($P < 0.01$) inhibited by over 90% the amounts of [^{3}H]-leucine incorporated into cellular and secreted proteins by Day-15 guinea-pig endometrium in culture for 24h (Figure 16).

Conclusions

Sodium fluoride (10mM) inhibited both cellular and secreted protein synthesis by guinea-pig endometrium in culture. Therefore the inhibition of PG output from guinea-pig endometrium in culture

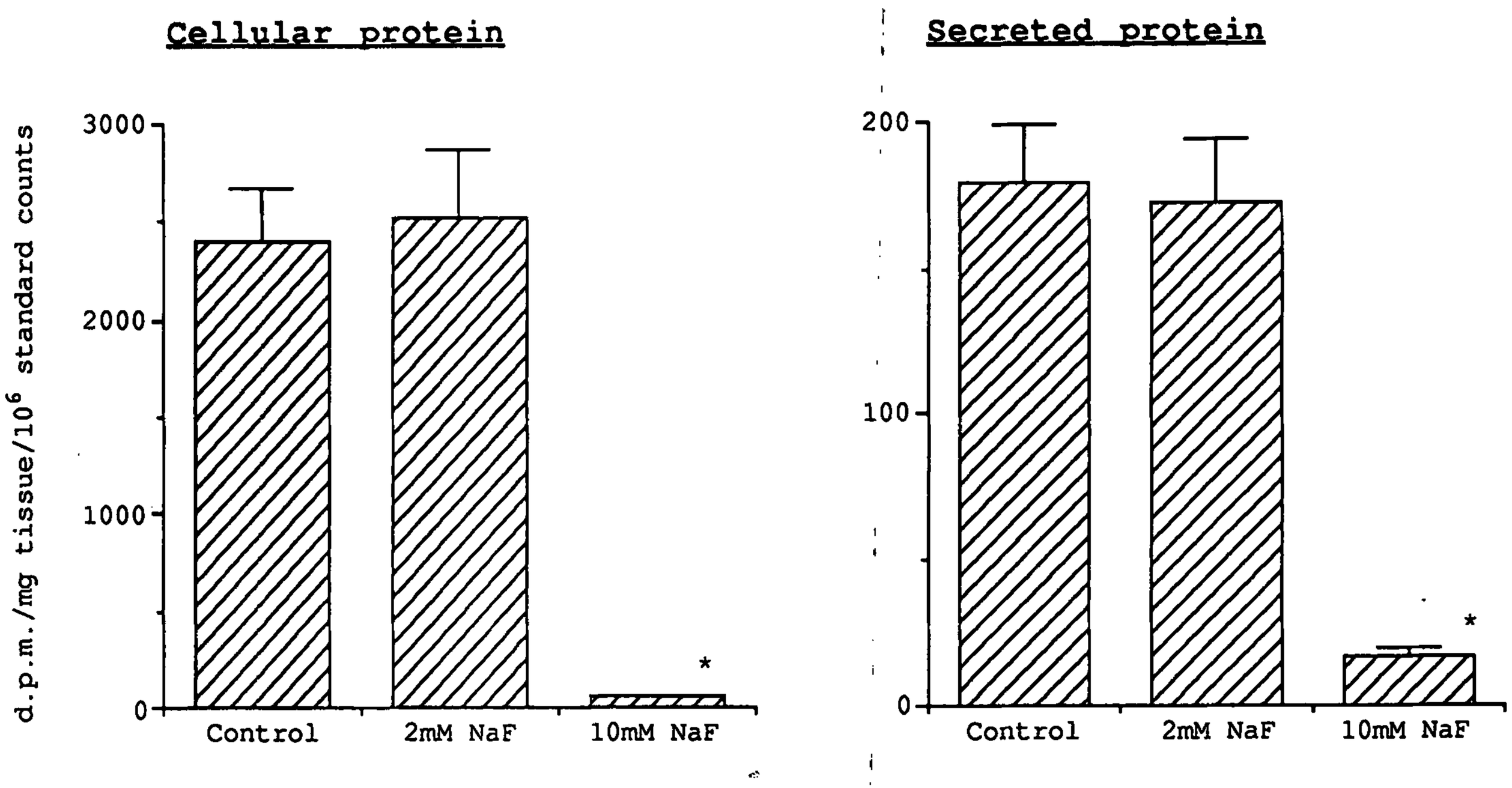


Fig. 16. Effects of sodium fluoride (NaF; 2 and 10mM) on mean (± s.e.m., n = 10) amounts of [^{3}H]-leucine incorporated into cellular and secreted proteins by Day-15 guinea-pig endometrium cultured for 24h.*Significantly ($P < 0.05$) lower than the corresponding control value for the same type of protein.

caused by sodium fluoride (Section 3:1:a) is probably a consequence of sodium fluoride-induced inhibition of protein synthesis.

3:1:c The effects of cholera toxin and sodium fluoride on the output of prostaglandins from guinea-pig endometrium in culture for 1 hour

Introduction

In Section 3:1:a sodium fluoride (10mM) had a tendency to stimulate PG production by Day-7 guinea-pig endometrium during the first 8h of culture. This stimulation was significant ($\underline{P} < 0.05$) for 6-keto-$PGF_{1\alpha}$ after 16h of culture. It was therefore decided to culture Day-7 guinea-pig endometrium for a shorter time period (1h) in order to investigate whether any significant stimulatory effects of sodium fluoride on PG production could be seen before the inhibitory effect of sodium fluoride on protein synthesis (see Section 3:1:b) became apparent and masked any short term stimulatory effects which might be occurring. The effect of cholera toxin on the output of PGs from the Day-7 guinea-pig endometrium in culture for 1h was also investigated to determine whether the toxin had an effect on PG output over a shorter time period.

Methods

The uteri were removed from five Day-7 guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 10 petri dishes containing 15-30mg wet weight (3-6mg dry weight) of endometrium were prepared from each uterus. Pairs of dishes were treated with 10ng/ml cholera toxin, 100ng/ml cholera toxin, 2mM sodium fluoride, 10mM sodium fluoride and no treatment (control). The petri dishes were incubated at 37°C for 1h. The samples of culture medium obtained

were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each petri dish into preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was then reweighed and the amount of dried endometrium from each petri dish was calculated. The outputs of PGs were calculated per mg dry weight of endometrium.

Statistical tests

Differences between treated and control groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t*-test for unequal variances.

Results

10mM sodium fluoride significantly ($P < 0.05$) increased the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium cultured for 1h (Figure 17). Cholera toxin (10 and 100ng/ml) and sodium fluoride (2mM) had no effect on endometrial PG synthesis except that 2mM sodium fluoride significantly ($P < 0.05$) increased the output of 6-keto-$PGF_{1\alpha}$ (Figure 17).

Conclusions

Sodium fluoride (10mM) stimulated the output of all 3 PGs from Day-7 guinea-pig endometrium in culture for 1h. Cholera toxin (10 and 100ng/ml) had no effect on PG output over 1h of culture. There may, therefore, be an intermediary G-protein stimulating PG synthesis which is fluoride-sensitive and toxin-insensitive.

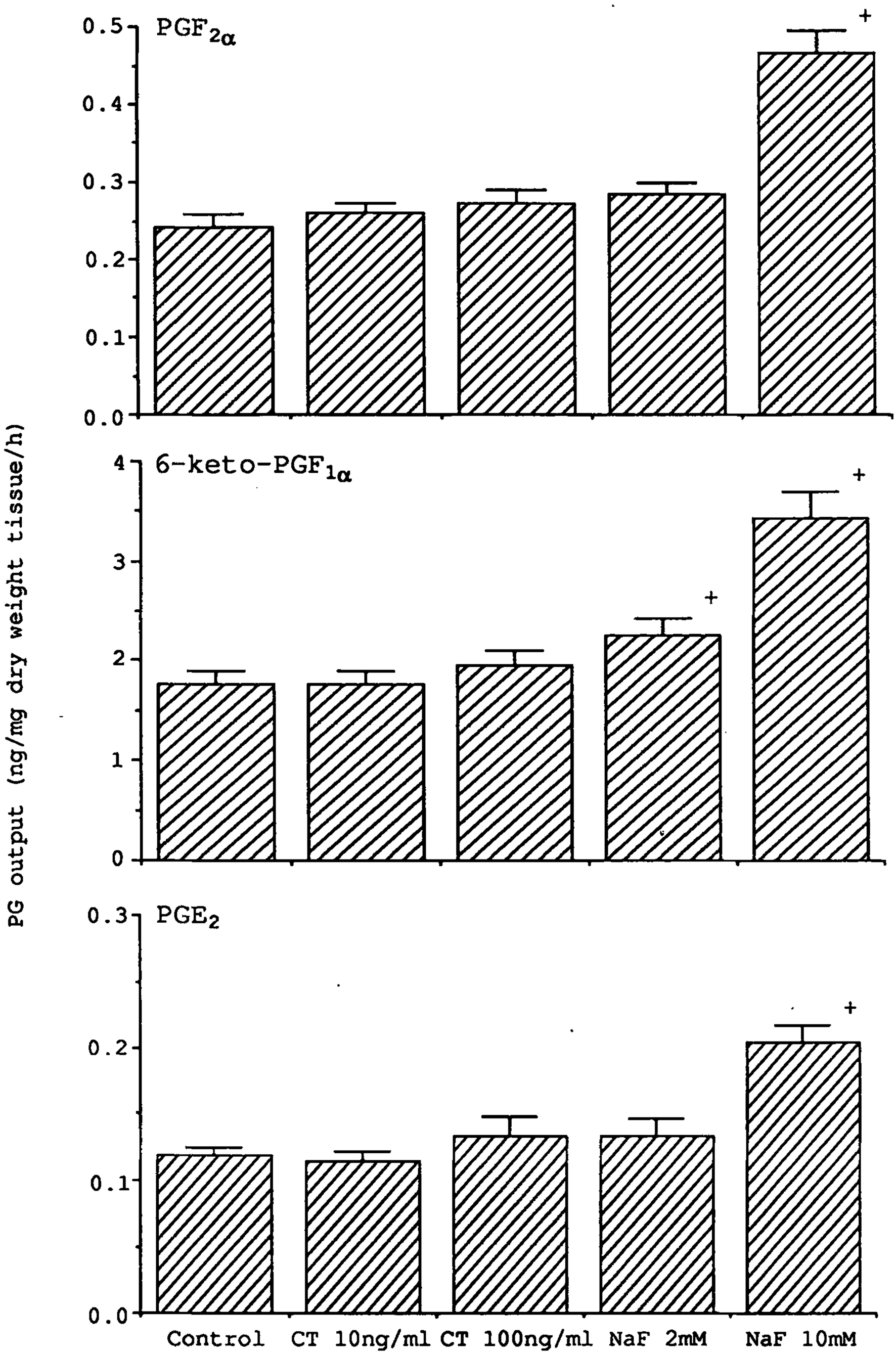


Fig. 17. Effects of cholera toxin (CT; 10 and 100ng/ml) and sodium fluoride (NaF; 2 and 10mM) on mean (± s.e.m., $n = 10$) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium cultured for 1h. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG.

DISCUSSION

The amounts of $PGF_{2\alpha}$ and, to a lesser extent, PGE_2 released into culture medium by guinea-pig endometrium were found to be higher on Day-15 than on Day-7 of the cycle, whereas the amounts of 6-keto-$PGF_{1\alpha}$ released were virtually unchanged between the two days. The degree of stimulation in the outputs of each PG from guinea-pig endometrium in culture between Day-7 and Day-15 of the cycle is in agreement with the findings of Riley and Poyser (1989, 1990) who also observed that the outputs of $PGF_{2\alpha}$ and PGE_2 from guinea-pig endometrium cultured for 24h were higher on Day-15 than on Day-7 of the cycle, while the release of 6-keto-$PGF_{1\alpha}$ remained almost the same. As PG metabolism by the guinea-pig uterus is low and no detectable metabolism occurs in uterine samples incubated without NAD^+ (Poyser, 1979), these differences are not due to changes in the metabolism of PGs. Therefore, these results show that the preferential stimulation of guinea-pig endometrial $PGF_{2\alpha}$ synthesis *in vivo* around Day 11 of the cycle (Blatchley *et al.*, 1972; Earthy *et al.*, 1975; Antonini *et al.*, 1976), which is caused by oestradiol acting on a progesterone- primed uterus (Blatchley and Poyser, 1974; Poyser, 1983a, 1983b), is maintained in tissue culture in the absence of steroids.

The stimulation of PG output from the guinea-pig endometrium by sodium fluoride over 1h and 8h of culture suggests that a G-protein may be involved in the control of endometrial PG synthesis in the guinea-pig. However, the lack of effect on endometrial PG synthesis at 1h, 8h, 16h and 24h of the G-protein modulators pertussis toxin and cholera toxin indicates that the G-protein is toxin-insensitive. The activation of PLA_2 and stimulation of PGE_2 formation by Swiss 3T3 fibroblast cells in response to bradykinin is also

mediated by a pertussis toxin-insensitive G-protein (Burch and Axelrod, 1987).

However, over longer periods of culture, sodium fluoride inhibited PG output from the guinea-pig endometrium, and this was probably due to an inhibition of protein synthesis. The effects of sodium fluoride are, therefore, similar to those of the protein synthesis inhibitors actinomycin D, cycloheximide and puromycin which also inhibit both endometrial protein and PG synthesis (Riley and Poyser, 1989). $PGF_{2\alpha}$ output from both the Day-7 and the Day-15 guinea-pig endometrium in culture is more sensitive to inhibition of protein synthesis by sodium fluoride than the outputs of 6-keto-$PGF_{1\alpha}$ or PGE_2. Therefore, it appears that $PGF_{2\alpha}$ synthesis is specifically dependent on fresh protein synthesis. Progesterone has also been shown to have an inhibitory effect on protein synthesis by the guinea-pig endometrium (Riley and Poyser, 1990), and thus the increase in the control output of $PGF_{2\alpha}$ from Day-7 endometrium over 24h of culture may be due to the removal of the tissue from the animal with the consequent loss of the inhibitory influence of progesterone, which is known to be present at high concentrations in plasma at this stage of the cycle (Joshi, Watson and Labhsetwar, 1973). It has previously been shown that ovariectomy in ewes on Day 12 results in higher concentrations of $PGF_{2\alpha}$ in the utero-ovarian vein on Day 13, and this effect is prevented by progesterone treatment (Vincent and Inskeep, 1986). Progesterone also inhibits the basal and oestradiol-stimulated outputs of $PGF_{2\alpha}$ from human endometrium maintained in culture (Cane and Villee, 1975; Abel and Baird, 1980; Kelly and Smith, 1987) and from human endometrial cells in culture (Skinner, Liggins, Wilson and Neale, 1984; Schatz, Markiewicz, Barg and Gurpide, 1985). In addition, progesterone

inhibits the output of $PGF_{2\alpha}$ from Day-7 and Day-15 guinea-pig endometrium in culture (Riley and Poyser, 1987b, 1989). This effect of progesterone is not due to a glucocorticoid-like activity of the steroid resulting in inhibition of PLA_2 activity by causing the synthesis of lipocortin (Flower, 1986), as the glucocorticoid, hydrocortisone, had no effect on PG output from the guinea-pig endometrium in culture (Riley and Poyser, 1987b). These results are in agreement with a study by Schatz, Markiewicz and Gurpide (1986) which found that, while dexamethasone and progesterone both reduced $PGF_{2\alpha}$ from human endometrium in culture, dexamethasone increased while progesterone decreased lipocortin output. Consequently, progesterone appears to inhibit the synthesis of a protein which specifically stimulates $PGF_{2\alpha}$ output from the endometrium and this effect of progesterone is mimicked by sodium fluoride and by other protein synthesis inhibitors in culture.

The production of 6-keto-$PGF_{1\alpha}$ by the guinea-pig endometrium was more sensitive to the stimulatory effects of sodium fluoride than that of $PGF_{2\alpha}$ and PGE_2, as it was the only PG to be significantly stimulated from the Day-7 and Day-15 endometrium by 10mM sodium fluoride during 24h of culture, and it was the only PG to be significantly stimulated from the Day-7 endometrium by the lower dose of sodium fluoride (2mM) over 1h of culture. It has been suggested that a different pool of arachidonic acid and a PLA_2 enzyme located in a different site are used for the production of 6-keto-$PGF_{1\alpha}$ than those which are used for the production of $PGF_{2\alpha}$ and PGE_2 (Poyser, 1991). Indeed, progesterone has been shown to inhibit the outputs of $PGF_{2\alpha}$ and PGE_2 from Day-7 and Day-15 guinea-pig endometrium in culture without affecting the output of 6-keto-$PGF_{1\alpha}$ (Riley and Poyser, 1987b). Similarly,

progesterone and a high concentration of oestradiol reduced the outputs of $PGF_{2\alpha}$, and to a lesser extent, of PGE_2 from Day-7 and Day-15 guinea-pig endometrium in culture, but had little or no effect on the output of 6-keto-$PGF_{1\alpha}$ (Riley and Poyser, 1990). Progesterone decreased and oestradiol increased the amounts of $PGF_{2\alpha}$ and PGE_2 synthesised by homogenates of Day-7 endometrium following culture of the endometrium in the presence of the steroids, but had no significant effect on the amounts of 6-keto-$PGF_{1\alpha}$ synthesised (Riley and Poyser, 1990). These findings indicate that the intracellular processes controlling PGI_2 synthesis (as reflected by measuring 6-keto-$PGF_{1\alpha}$ output) are different from those controlling the synthesis of $PGF_{2\alpha}$ and PGE_2. In particular, PGI_2 synthesis does not appear to depend on protein synthesis as at no time did sodium fluoride inhibit 6-keto-$PGF_{1\alpha}$ output from the guinea-pig endometrium. In addition the protein synthesis inhibitors -actinomycin D, cycloheximide and puromycin did not affect the endometrial output of 6-keto-$PGF_{1\alpha}$ (Riley and Poyser, 1989).

3:2 INVESTIGATIONS INTO THE MECHANISM BY WHICH SODIUM FLUORIDE STIMULATES PROSTAGLANDIN OUTPUT FROM THE GUINEA-PIG UTERUS

3:2:a The effect of sodium fluoride on prostaglandin output from the superfused guinea-pig uterus

Introduction

The previous study (section 3:1:c) showed that sodium fluoride was capable of stimulating the output of PGs from Day-7 endometrium in culture over the short term. The following experiments have therefore investigated the intracellular mechanisms involved in the stimulation of PG production by sodium fluoride by using an *in vitro* superfusion system developed by Poyser and Brydon (1983). Initially, the effects of sodium fluoride on the output of PGs from the Day-7 and the Day-15 uterus superfused *in vitro* were examined.

Methods

The uteri from five Day-7 and five Day-15 guinea-pigs were removed and separated into two horns. Each uterine horn was weighed, "opened" by a longitudinal incision, and superfused with Krebs' solution, preaerated with 5% CO_2 and 95% O_2, at 37°C at a rate of 5ml/min. Each horn was superfused initially for a "settling period" of 60 min and the samples of superfusate were collected for 10-min periods over the next 90 min (see Section 2:4). Sodium fluoride (10mM) was present in the Krebs' solution during the collection of samples 4, 5, and 6 from one uterine horn. The other uterine horn from each guinea-pig acted as the "control" in order to measure basal PG output during the period of superfusion. After collection, PGs were solvent extracted from the samples of superfusate (see Section 2:4) and were stored in 10ml ethyl acetate

at -20°C. The amounts of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 present were measured by radioimmunoasssy (see Section 2:9). The outputs of PGs were calculated per 100mg wet weight of uterus.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's multiple range test or, if the variances of the groups were significantly different by the variance ratio F test, by a modified t test for unequal variances. Differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

The initial outputs (i.e. during the first 30 min of sample collection) of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were 76.9-, 5.6- and 1.6-fold higher respectively, from the Day-15 uterus than from the Day-7 uterus, showing that the Day-15 uterus synthesises $PGF_{2\alpha}$ preferentially (Figures 18 and 19). Sodium fluoride (10mM) significantly ($P < 0.05$) increased the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the Day-7 and Day-15 guinea-pig uterus, and of PGE_2 from the Day-15 uterus (Figures 18 and 19). The output of PGE_2 from the Day-7 uterus tended to increase following sodium fluoride treatment, and was significantly ($P < 0.05$) higher than the output of PGE_2 from the control horn (Figure 18). The increases in outputs of $PGF_{2\alpha}$ and PGE_2 from Day-7 and Day-15 and of 6-keto-$PGF_{1\alpha}$ from the Day-15 uterus occurred slowly, and the maximum outputs were not reached until the third or fourth 10-min period of sample collection after starting the sodium fluoride

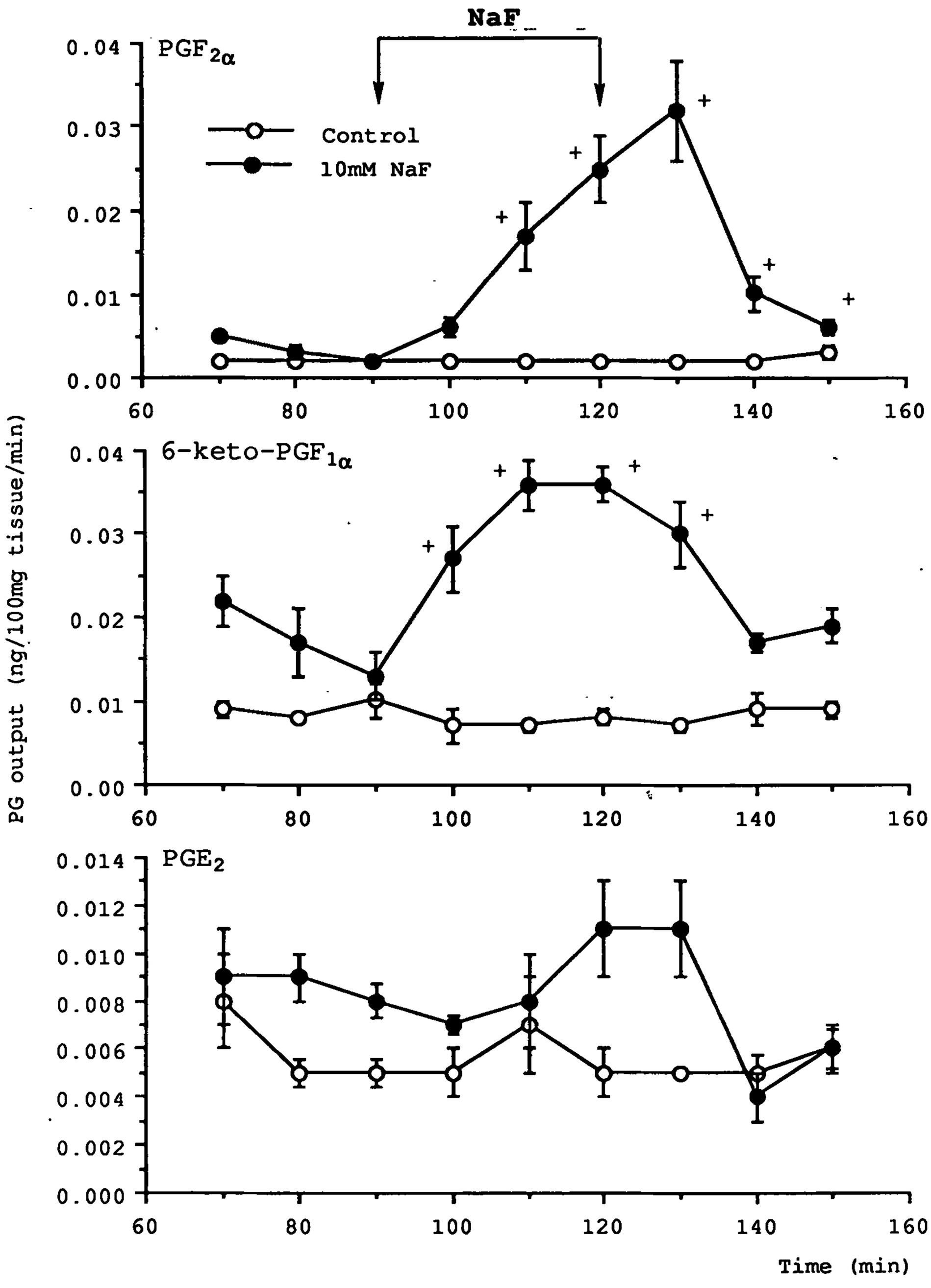


Fig. 18. Effect of sodium fluoride (NaF; 10mM, closed circles) on mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused *in vitro*. +Significantly ($P < 0.05$) higher than before sodium fluoride treatment.

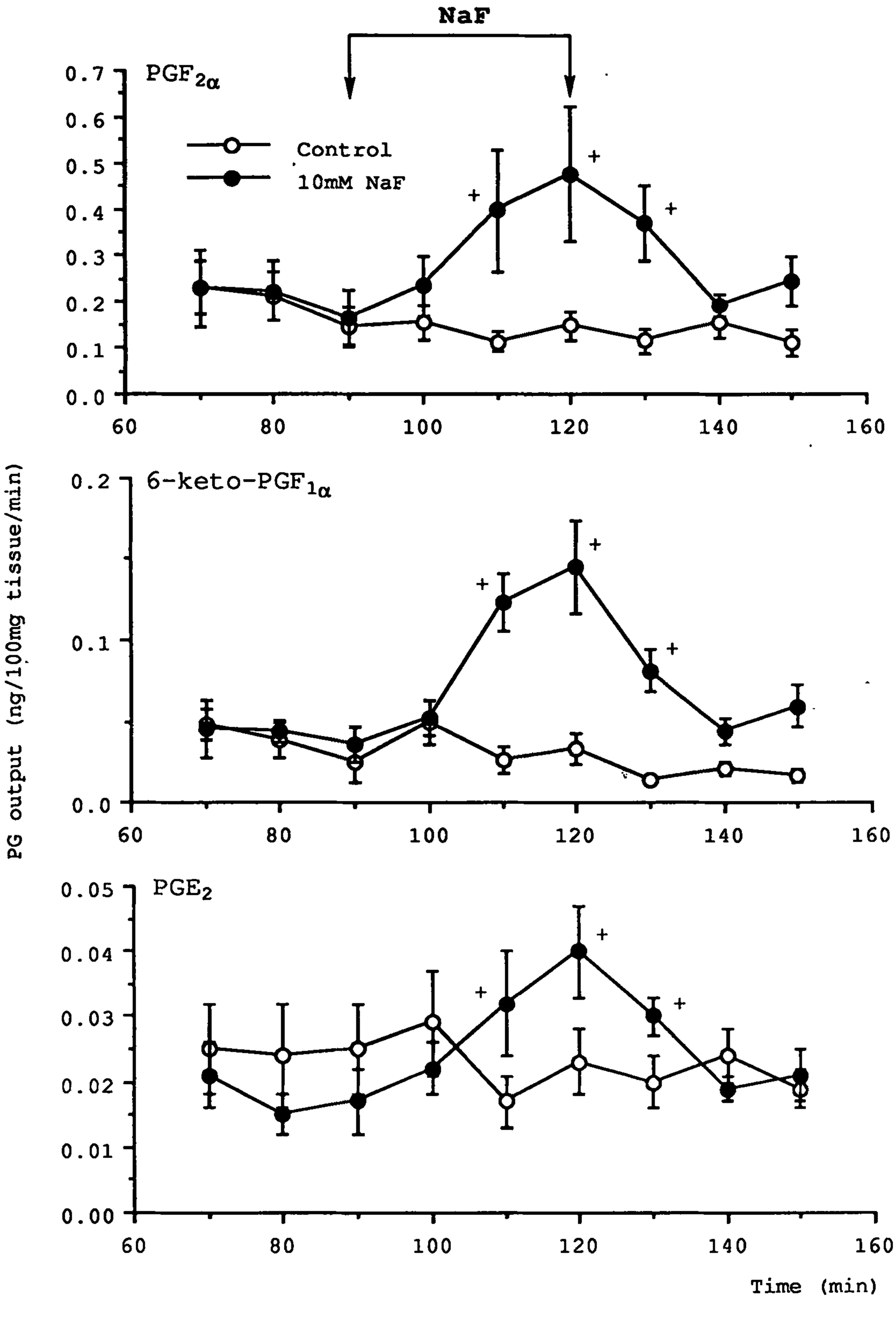


Fig. 19. Effect of sodium fluoride (NaF; 10mM, closed circles) on mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-15 guinea-pig uterus superfused *in vitro*. +Significantly ($P < 0.05$) higher than before sodium fluoride treatment.

treatment (Figures 18 and 19). However, the rise in 6-keto-$PGF_{1\alpha}$ output from the Day-7 uterus increased more rapidly, showing a significant ($P < 0.05$) increase during the first 10-min period and reaching a maximum during the second 10-min period after starting the sodium fluoride treatment (Figure 18).

Conclusions

Sodium fluoride stimulated the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and, to a lesser extent, PGE_2 from the Day-7 and Day-15 guinea-pig uterus superfused *in vitro*. The increase in uterine PG output caused by sodium fluoride was characterised by a lag period of 10-20 min, except in the case of the stimulation of 6-keto-$PGF_{1\alpha}$ output from the Day-7 uterus which was significant within 10 min. The involvement of calcium in the sodium fluoride-induced stimulation of PG output from the Day-7 uterus was investigated in the following experiment.

3:2:b The effects of lack of extracellular calcium and of the intracellular calcium antagonist TMB-8 on the sodium fluoride-stimulated output of prostaglandins from the superfused guinea-pig uterus

Introduction

Previous studies have shown that PC and PE, but not PI, are the probable sources of arachidonic acid for increased endometrial $PGF_{2\alpha}$ synthesis at the end of the cycle (Ning et al., 1983; Ning and Poyser, 1984), implicating the involvement of PLA_2 which is a calcium-dependent enzyme. Indeed, the calcium ionophore A23187 stimulates PG output from the guinea-pig uterus superfused in vitro (Poyser and Brydon, 1983) by an action dependent upon extracellular Ca^{2+} (Poyser, 1984b). Raising free intracellular Ca^{2+} concentrations is therefore able to stimulate arachidonic acid release for PG synthesis in the guinea-pig uterus. Since guinea-pig uterine microsomal PLA_2 is maximally active in 7mM Ca^{2+} (Downing and Poyser, 1983), which is several fold higher than physiological Ca^{2+} concentrations, increasing the free intracellular Ca^{2+} concentration is probably of great importance in activating PLA_2. Removal of the extracellular calcium, by using Krebs' solution from which the calcium chloride has been omitted, completely prevents the increases in outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the superfused guinea-pig uterus produced by A23187 (Poyser, 1984b). The compound TMB-8, which blocks the release of intracellular bound calcium (Malagodi and Chiou, 1974), also prevents the A23187-induced increase in PG output from the superfused Day-7 and Day-15 guinea-pig uterus in vitro (Poyser, 1985b). Therefore, the effects of lack of extracellular calcium and of the intracellular calcium

antagonist TMB-8 on the stimulation of PG output from the superfused guinea-pig uterus caused by sodium fluoride were examined.

Methods

The uteri were removed from five Day-7 guinea-pigs and separated into two horns. Each uterine horn was weighed, "opened" by a longitudinal incision, and superfused with Krebs' solution, preaerated with 5% CO_2 and 95% O_2, at 37°C at a rate of 5ml/min. Each horn was superfused initially for a "settling period" of 60 min and the samples of superfusate were collected for 10-min periods over the next 90 min (see Section 2:4). The Krebs' solution superfusing one uterine horn from each of the five Day-7 guinea-pigs lacked calcium chloride (i.e. calcium-free Krebs' solution), and sodium fluoride (10mM) was added to the solution superfusing both uterine horns during the collection of samples 4, 5 and 6. Samples of superfusate were collected for 90 min.

For another five Day-7 guinea-pigs, the intracellular calcium antagonist TMB-8 (150μM) was present in the Krebs' solution superfusing one uterine horn during the collection of samples 4-8. In addition 10mM sodium fluoride was present in the normal Krebs' solution bathing both uterine horns during the collection of samples 6, 7 and 8. Samples of superfusate were collected for 110 min.

After collection, PGs were solvent extracted from the samples of superfusate (see Section 2:4) and were stored in 10ml ethyl acetate at -20°C. The amounts of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 present were measured by radioimmunoasssy (see Section 2:9). The outputs of PGs were calculated per 100mg wet weight of uterus.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's multiple range test or, if the variances of the groups were significantly different by the variance ratio F test, by a modified t test for unequal variances. Differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

The use of calcium-free Krebs' had no significant effect on the sodium fluoride-stimulated increases in the outputs of $PGF_{2\alpha}$ and PGE_2 from the Day-7 uterus. However, the use of calcium-free Krebs' solution delayed the onset of the increase in 6-keto-$PGF_{1\alpha}$ output produced by sodium fluoride and also significantly ($P < 0.05$) decreased the maximum output of 6-keto-$PGF_{1\alpha}$ obtained (Figure 20).

TMB-8 (150μM) alone caused a small, sometimes significant ($P < 0.05$) increase in the outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 uterus (Figure 21). TMB-8 prevented the increases in the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ produced by sodium fluoride. However, the output of 6-keto-$PGF_{1\alpha}$ significantly ($P < 0.05$) increased to a small extent after the combined superfusion of TMB-8 and sodium fluoride had finished.

Conclusions

The removal of extracellular calcium from the Krebs' solution had no effect on the stimulation of $PGF_{2\alpha}$ and PGE_2 outputs produced by sodium fluoride from the Day-7 guinea-pig uterus. This indicates that the action of sodium fluoride in stimulating the synthesis of

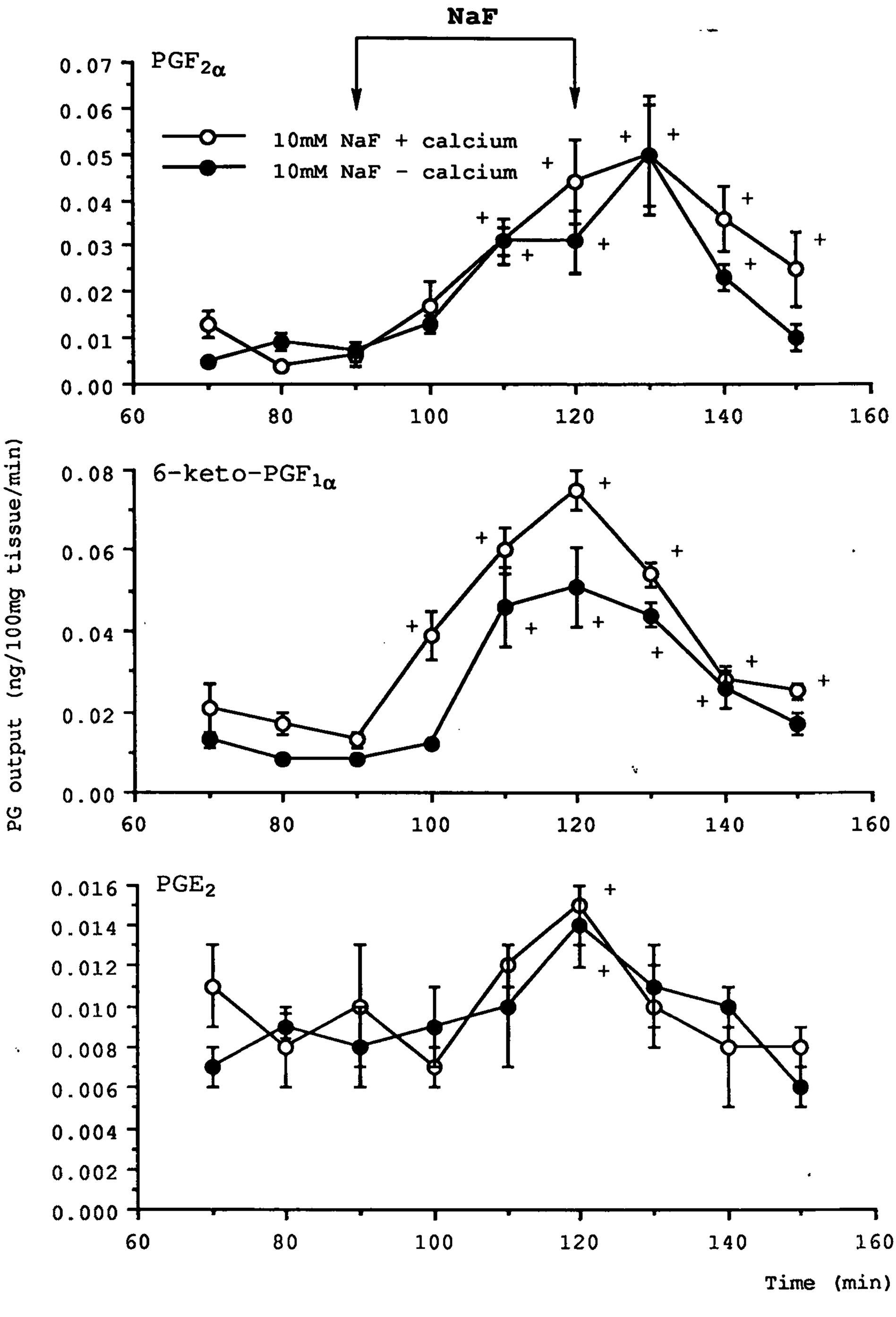


Fig. 20. Effect of lack of calcium (closed circles) on sodium fluoride (NaF)-stimulated mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused in vitro. +Significantly ($P < 0.05$) higher than before sodium fluoride treatment.

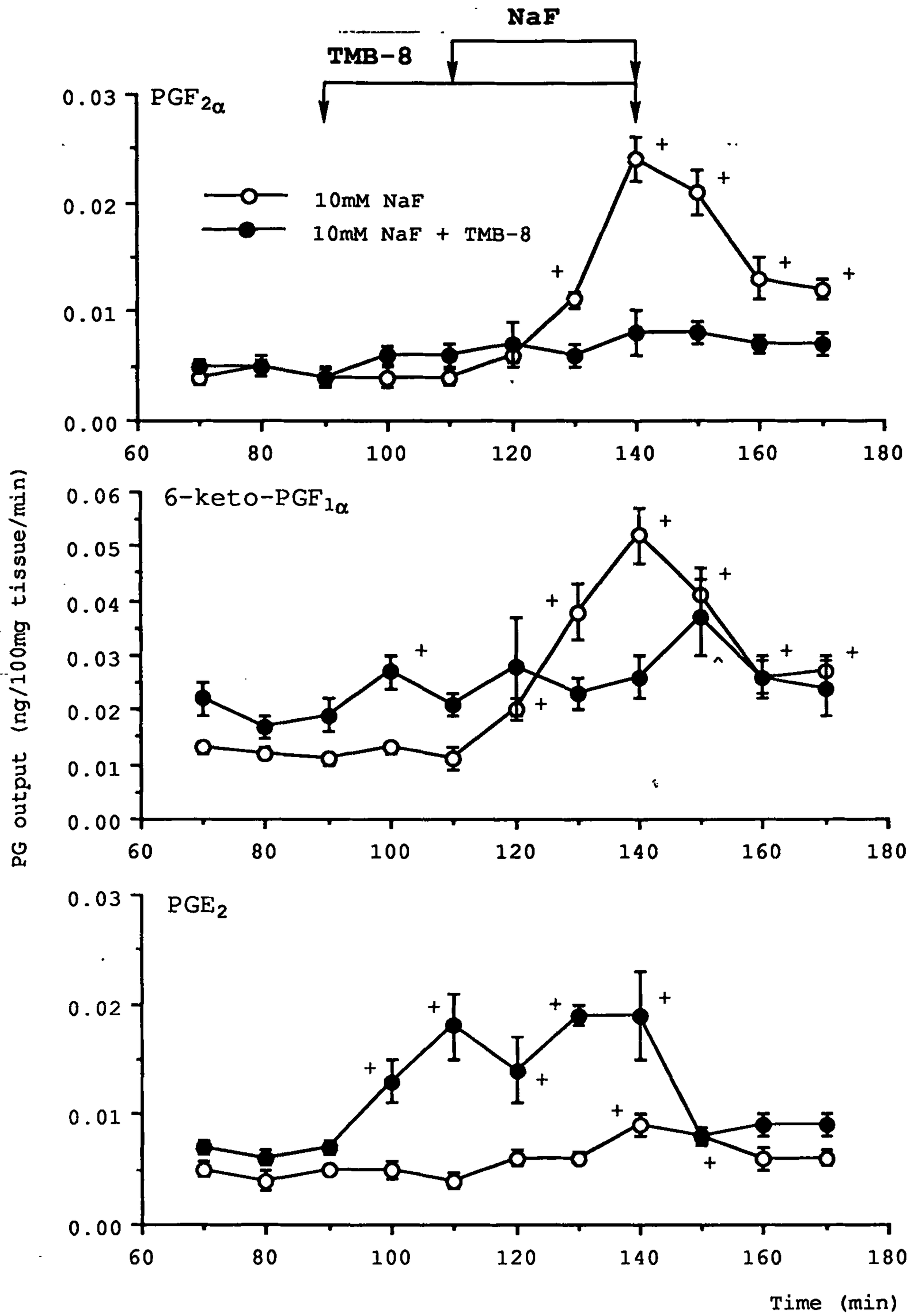


Fig. 21. Effect of TMB-8 (150μM; closed circles) on sodium fluoride (NaF)-stimulated mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused in vitro. +Significantly ($P < 0.05$) higher than before sodium fluoride treatment alone. ^Significantly ($P < 0.05$) higher than before any treatment and TMB-8 treatment alone.

these two PGs by the guinea-pig uterus is not dependent upon extracellular calcium.

However, the initial, quicker rise in 6-keto-$PGF_{1\alpha}$ output was prevented and the maximum output of 6-keto-$PGF_{1\alpha}$ was reduced after stimulation by sodium fluoride of the Day-7 guinea-pig uterus superfused with calcium-free Krebs' solution. This indicates that part of the response to sodium fluoride in the stimulation of 6-keto-$PGF_{1\alpha}$ synthesis by the Day-7 guinea-pig uterus is dependent upon extracellular calcium.

The intracellular calcium antagonist, TMB-8, caused a small stimulation of 6-keto-$PGF_{1\alpha}$ output and a larger, more prolonged stimulation of PGE_2 output from the guinea-pig uterus. TMB-8 prevented the increases in outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the Day-7 guinea-pig uterus produced by sodium fluoride. These findings indicate that the stimulation of $PGF_{2\alpha}$ and PGE_2 synthesis and the second component in the stimulation of 6-keto-$PGF_{1\alpha}$ synthesis by sodium fluoride in the guinea-pig uterus are dependent upon intracellular calcium.

3:2:c - The effects of the calmodulin antagonists trifluoperazine and W-7 on the sodium fluoride-stimulated output of prostaglandins from the superfused guinea-pig uterus

Introduction.

The study in Section 3:2:b has shown that the increase in uterine PG synthesis caused by sodium fluoride is probably due to the release of intracellular calcium. Many of the effects of Ca^{2+} are mediated through calmodulin-regulated enzymes (Cheung, 1980). Calmodulin is a small acidic protein (Mr 16,700 Da) consisting of a single polypeptide chain with four loops, each loop possessing a domain for binding a calcium ion. Calmodulin plays an important role in the activation and regulation of many Ca^{2+}-dependent processes in eukaryotic cells including the control of several enzyme activities. PLA_2 is a calmodulin-dependent enzyme in several tissues (Wong and Cheung, 1979; Moskowitz, Shapiro, Schook and Puszkin, 1983) and the calmodulin antagonists, trifluoperazine (Levin and Weiss, 1977) and W-7 (Hidaka, Asano, Iwadare, Matsumoto, Totsuka and Aoki, 1978), inhibit the A23187-induced increases in outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from the guinea-pig uterus superfused *in vitro* (Poyser, 1985a, 1985b). Therefore, in order to examine whether calmodulin is involved in mediating the increase in uterine PG synthesis produced by sodium fluoride, the effects of trifluoperazine and W-7 on the stimulation of PG output from the Day-7 guinea-pig uterus caused by sodium fluoride were examined.

Methods

The uteri from five Day-7 guinea-pigs were removed and separated into two horns. Each uterine horn was weighed, "opened" by a

longitudinal incision, and superfused with Krebs' solution, preaerated with 5% CO_2 and 95% O_2, at 37°C at a rate of 5ml/min. Each horn was superfused initially for a "settling period" of 60 min and the samples of superfusate were collected for 10-min periods over the next 110 min (see Section 2:4). 100μM trifluoperazine was present in the Krebs' solution superfusing one uterine horn from five Day-7 guinea-pigs during the collection of samples 4-8. In addition sodium fluoride (10mM) was present in the Krebs' solution bathing both uterine horns during the collection of samples 6, 7 and 8.

The above experiment was repeated for another five Day-7 guinea-pigs using 150μM W-7 in place of trifluoperazine. After collection, PGs were solvent extracted from the samples of superfusate in both experiments (see Section 2:4) and were stored in 10ml ethyl acetate at -20°C. The amounts of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 present were measured by radioimmunoasssy (see Section 2:9). The outputs of PGs were calculated per 100mg wet weight of uterus.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's multiple range test or, if the variances of the groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances. Differences between treated and control groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

Trifluoperazine (100μM) and W-7 (150μM) caused a small, sometimes significant ($P < 0.05$) increase in the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 uterus (Figures 22 and 23). Neither trifluoperazine or W-7 prevented the increases in the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 produced by sodium fluoride (Figures 22 and 23).

Conclusions

The calmodulin antagonists, trifluoperazine and W-7, had no inhibitory effect on the stimulation of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 outputs from the Day-7 guinea-pig uterus. Therefore, it appears that calmodulin (or a related compound) is not involved in the stimulation of uterine PG synthesis caused by sodium fluoride.

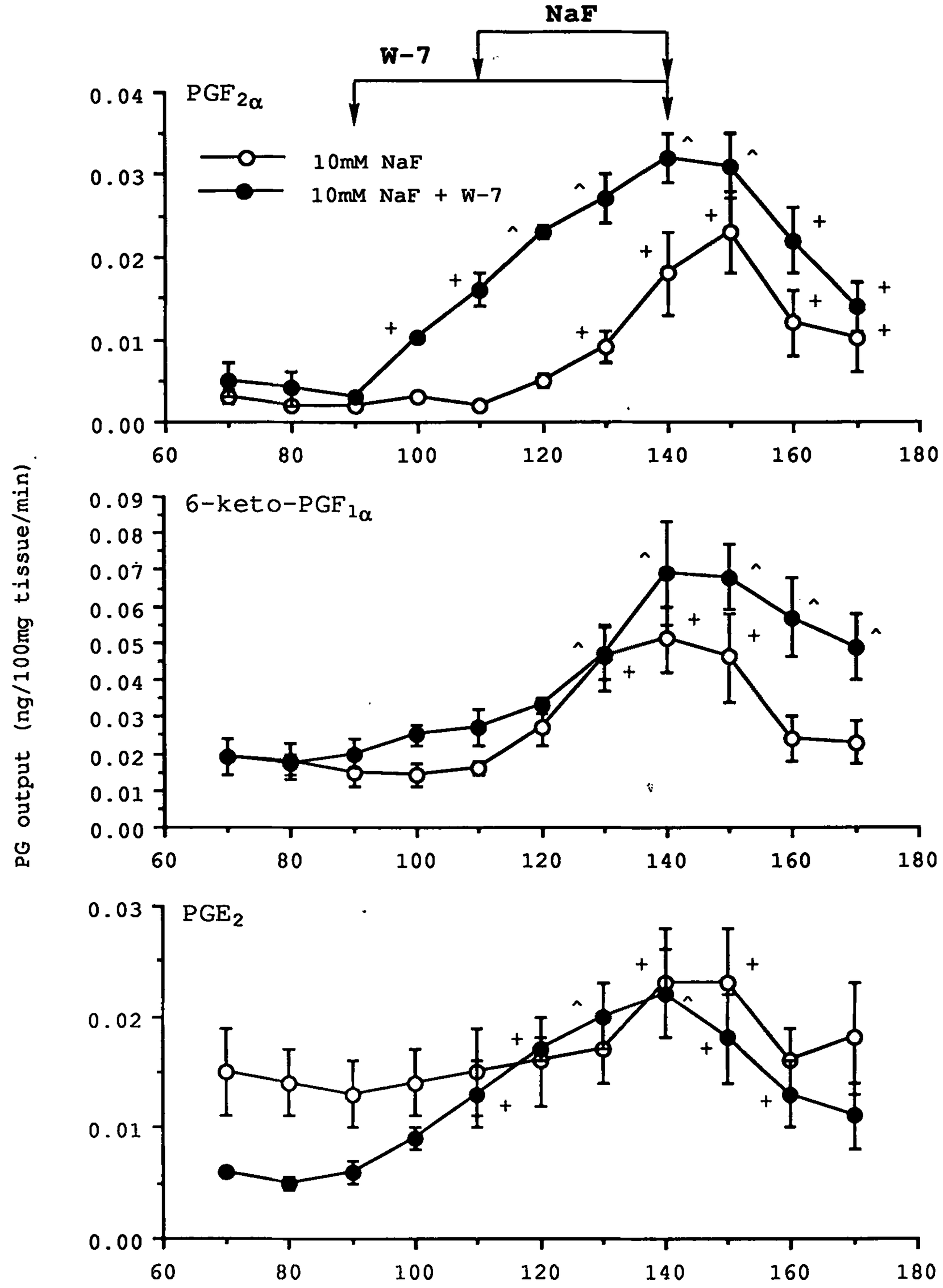


Fig. 22. Effect of W-7 (150μM; closed circles) on sodium fluoride (NaF)-stimulated mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused *in vitro*. +Significantly ($P < 0.05$) higher than before any treatment. ^Significantly ($P < 0.05$) higher than before any treatment and W-7 treatment alone.

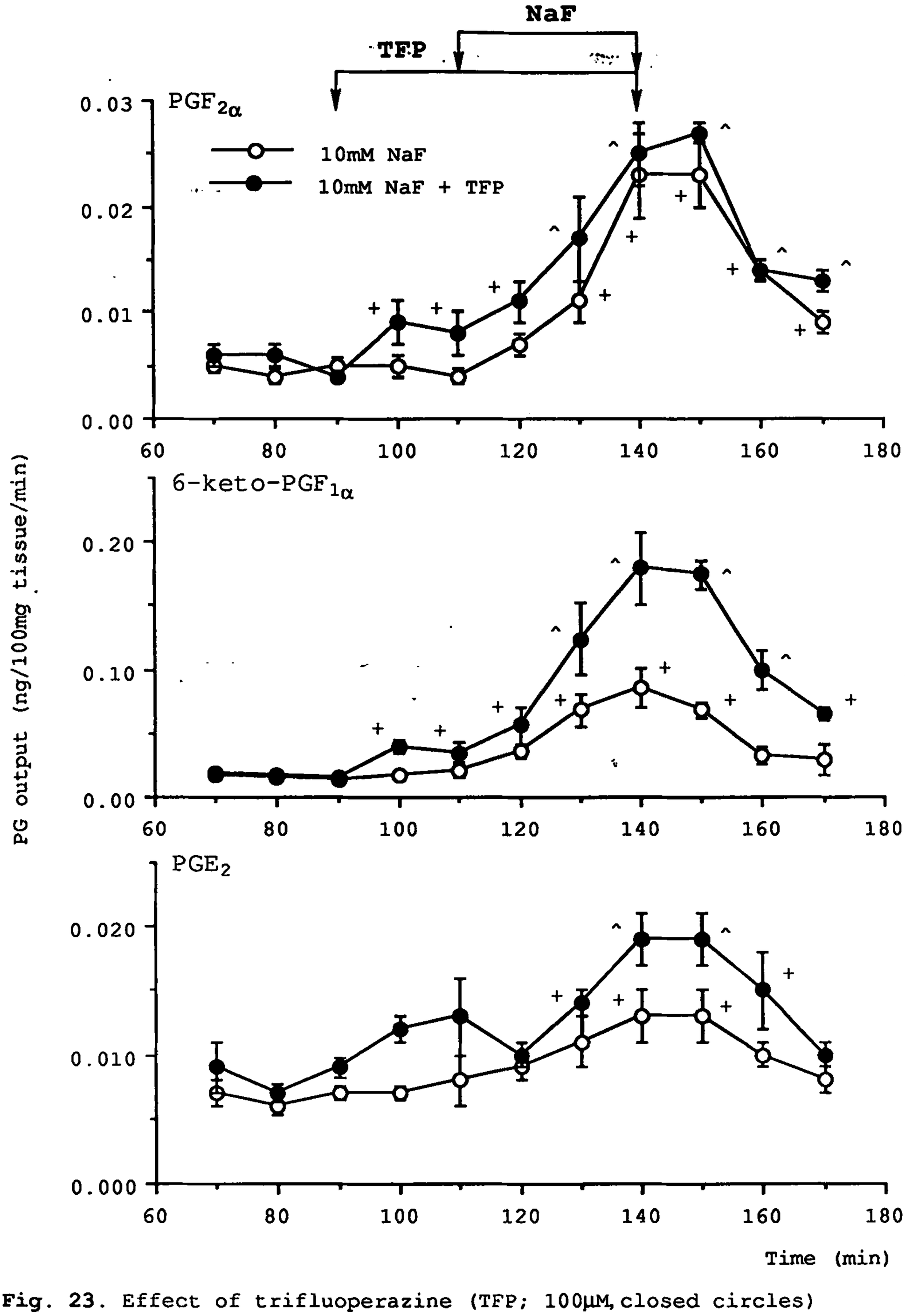


Fig. 23. Effect of trifluoperazine (TFP; 100μM, closed circles) on sodium fluoride (NaF)-stimulated mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused *in vitro*. +Significantly ($P < 0.05$) higher than before sodium fluoride treatment alone. ^Significantly ($P < 0.05$) higher than before any treatment and TFP treatment alone.

3:2:d The effect of the PLC inhibitor neomycin on the sodium fluoride-stimulated output of prostaglandins from the superfused guinea-pig uterus

Introduction

Stimulation of the enzyme PLC causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) which results in the generation of two intracellular messengers, namely diacylglycerol (DAG), which activates protein kinase C (Nishizuka, 1984), and inositol 1,4,5-trisphosphate (IP_3). IP_3 causes the release of Ca^{2+} from intracellular pools (Streb, Irvine, Berridge and Schulz, 1983). Therefore, sodium fluoride-stimulated PG synthesis may be due to activation of PLC and the formation of IP_3, which leads to increased intracellular Ca^{2+} levels and activation of PLA_2. In addition, if sodium fluoride stimulates PG synthesis by the activation of a fluoride-sensitive, toxin-insensitive (see Sections 3:1:a and 3:1:c) G-protein, it is possible that the G-protein could be linked to PLC and not to PLA_2. Therefore, the effect of the PLC inhibitor neomycin (Orsukalova, Stockhorst and Schacht, 1976; Schacht, 1976) was examined on the sodium fluoride-stimulated increase in PG output from the Day-7 guinea-pig uterus superfused *in vitro*.

Methods

The uteri from five Day-7 guinea-pigs were removed and separated into two horns. Each uterine horn was weighed, "opened" by a longitudinal incision, and superfused with Krebs' solution, preaerated with 5% CO_2 and 95% O_2, at 37°C at a rate of 5ml/min. Each horn was superfused initially for a "settling period" of 60 min

and the samples of superfusate were collected for 10-min periods over the next 110 min (see Section 2:4). 1mM neomycin sulphate was present in the Krebs' solution superfusing one uterine horn from each of the five Day-7 guinea-pigs during the collection of samples 4-8. In addition, 10mM sodium fluoride was present in the Krebs' solution bathing both uterine horns during the collection of samples 6, 7 and 8.

After collection, PGs were solvent extracted from the samples of superfusate (see Section 2:4) and were stored in 10ml ethyl acetate at -20°C. The amounts of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 present were measured by radioimmunoasssy (see Section 2:9). The outputs of PGs were calculated per 100mg wet weight of uterus.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's multiple range test or, if the variances of the groups were significantly different by the variance ratio F test, by a modified t test for unequal variances. Differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

Neomycin sulphate (1mM) had no effect on the basal or sodium fluoride-stimulated outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 (Figure 24).

Conclusions

Neomycin had no effect on the increases in outputs of $PGF_{2\alpha}$,

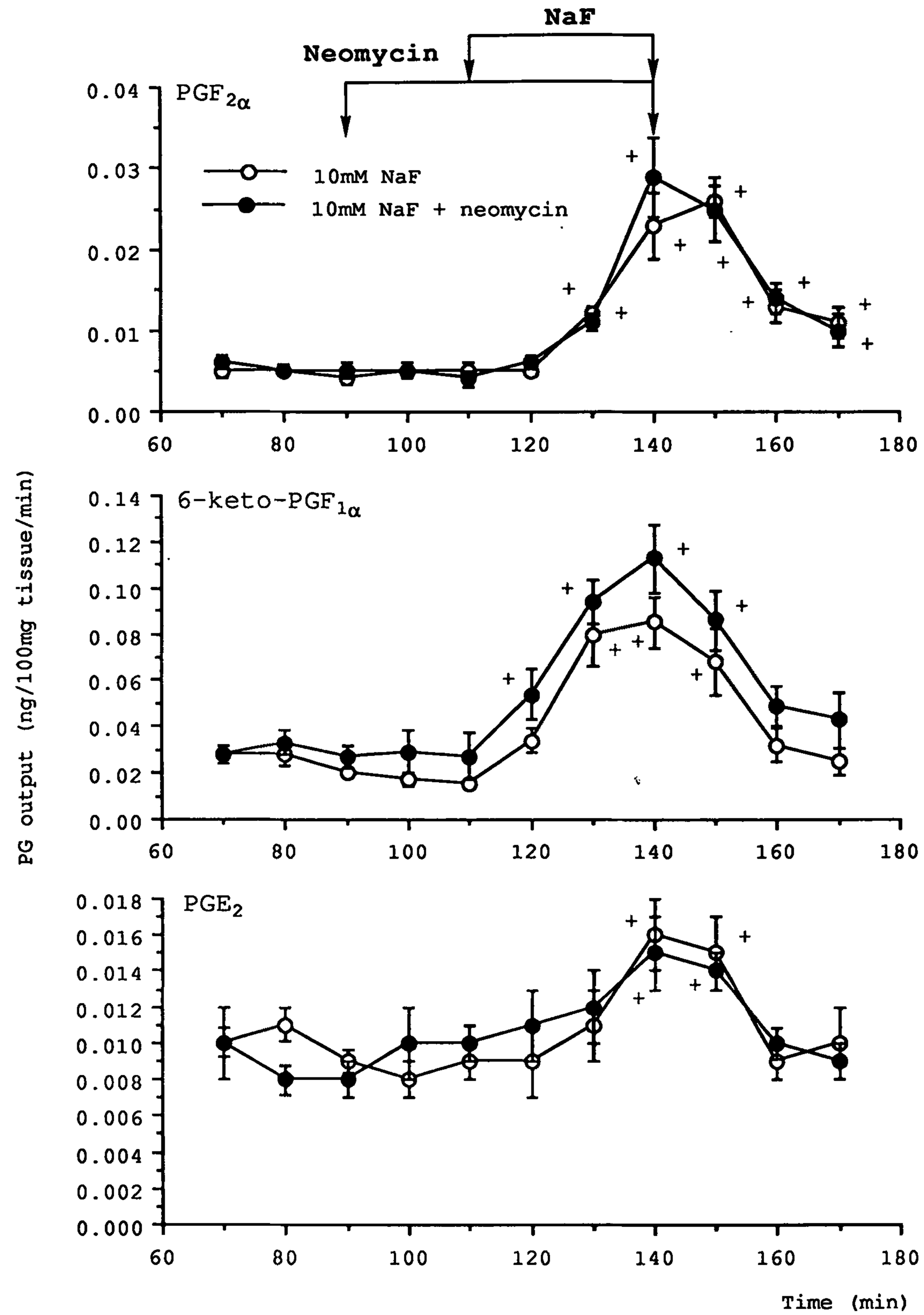


Fig. 24. Effect of neomycin (1mM; closed circles) on sodium fluoride (NaF)-stimulated mean (± s.e.m., n = 5) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus superfused *in vitro*.+Significantly ($P < 0.05$) higher than before sodium fluoride treatment.

6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 guinea-pig uterus produced by sodium fluoride. Thus it is unlikely that sodium fluoride stimulates endometrial PG synthesis by activating PLC.

DISCUSSION

Sodium fluoride stimulated the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ and, to a lesser extent, PGE_2 from the Day-7 and Day-15 guinea-pig uterus superfused in vitro. A similar pattern of stimulation of PG production was caused by the calcium ionophore A23187 when superfused over the guinea-pig uterus (Poyser and Brydon, 1983; Poyser, 1985a, 1985b, 1991). However, whereas the peak stimulation of output of all three PGs occurred within the first 10-min period of A23187 treatment, the peak stimulation of $PGF_{2\alpha}$ and PGE_2 outputs from Day-7 and Day-15 uterus and of 6-keto-$PGF_{1\alpha}$ from the Day-15 uterus did not occur until the third or fourth 10-min period of sodium fluoride treatment. Therefore, sodium fluoride causes a slower stimulation of uterine PG synthesis than A23187. However, an exception was the stimulation by sodium fluoride of 6-keto-$PGF_{1\alpha}$ output from the Day-7 uterus where a significant increase was observed in the first 10-min period and the maximum increase in output occurred during the second 10-min period of sodium fluoride treatment.

The sodium fluoride-stimulated increase in the output of PGs from the Day-7 uterus was unaffected by the removal of calcium from the superfusing solution. This is in contrast to the effect of lack of extracellular calcium on the A23187-induced increase in PG output from the guinea-pig uterus, which is prevented by removal of calcium from the superfusing solution (Poyser, 1984b). However, lack of extracellular calcium did prevent the initial quick rise in

6-keto-$PGF_{1\alpha}$ output and did reduce the maximum output produced by sodium fluoride. In a previous study (Poyser, 1984b), it was found that using calcium-free Krebs' solution significantly reduced by 50% the basal output of 6-keto-$PGF_{1\alpha}$ but had no effect on the basal outputs of $PGF_{2\alpha}$ and PGE_2 from the superfused Day-15 guinea-pig uterus. Consequently, part of the basal increase in 6-keto-$PGF_{1\alpha}$ production by the Day-15 guinea-pig uterus is dependent upon extracellular calcium. This increase appears to be maximal since sodium fluoride could not produce any further stimulation of the extracellular calcium-dependent synthesis of 6-keto-$PGF_{1\alpha}$ (i.e. there was no initial fast increase in the output of 6-keto-$PGF_{1\alpha}$ from the Day-15 guinea-pig uterus superfused in vitro). However, the slower stimulation of 6-keto-$PGF_{1\alpha}$ synthesis by sodium fluoride was still produced in the Day-15 guinea-pig uterus.

TMB-8 caused a small stimulation of 6-keto-$PGF_{1\alpha}$ output and a larger, more sustained stimulation of PGE_2 output from the Day-7 guinea-pig uterus superfused in vitro. These phenomena were noted previously (Poyser, 1985b), particularly the more prolonged stimulation of PGE_2 output, and is probably due to TMB-8 releasing small quantities of intracellular calcium (Pian-Smith, Yada, Yaney, Abdel-el-Motal, Wiedenkeller and Sharp, 1988; Taylor and Clark, 1988). TMB-8 prevented the increases in $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the Day-7 uterus caused by sodium fluoride. This indicates that sodium fluoride may stimulate PG synthesis by mobilising calcium from intracellular stores.

The high basal output of $PGF_{2\alpha}$ from Day-15 guinea-pig uterus superfused in vitro is not reduced during superfusion with calcium-free medium (Poyser, 1984b) or during a superfusion with TMB-8 (Poyser, 1985b). This indicates that, once stimulated in

vivo, PLA_2 continues to be activated for several hours in vitro. However, longer use of calcium-depleted medium or of TMB-8 for several days in culture reduced the basal output of $PGF_{2\alpha}$ from the Day-15 endometrium (Riley and Poyser, 1987a). As the removal of extracellular calcium did not affect the stimulation of PG output from the Day-7 uterus caused by sodium fluoride or the basal output of PGs from the Day-15 uterus (Poyser, 1984b), the action of sodium fluoride may mimic the mechanism by which oestradiol and progesterone stimulate uterine PG synthesis at the end of the cycle in vivo. However, TMB-8 did inhibit the sodium fluoride-induced increase in PG synthesis from the Day-7 superfused guinea-pig uterus unlike its lack of an inhibitory effect on the basal output of PGs from the Day-15 superfused guinea-pig uterus (Poyser, 1985b). This difference would be expected if sodium fluoride is able to act by stimulating a protein which mobilises intracellular calcium in the Day-7 uterus, but is unable to stimulate such a mechanism in the Day-15 uterus due to this mechanism already having been switched on in vivo by oestradiol acting on a progesterone-primed uterus from Day-11 of the cycle.

The small stimulation of PG production from the Day-7 guinea-pig uterus caused by trifluoperazine or W-7 alone has been noted previously (Poyser, 1985a, 1985b), and is probably due to these compounds displacing membrane-bound calcium (Seeman, 1972) which in turn, activates PLA_2 causing arachidonic acid release (Takenawa, Homma and Nagai, 1982) and PG synthesis. Neither trifluoperazine nor W-7 inhibited the sodium fluoride-induced increases in the outputs of PGs from the superfused Day-7 uterus. Therefore, calmodulin may not be involved in the mechanism by which intracellular calcium stimulates uterine $PGF_{2\alpha}$ production. This result again suggests

that sodium fluoride may act via a mechanism similar to oestradiol acting on a progesterone-primed uterus, as W-7 and trifluoperazine do not inhibit the high basal output of $PGF_{2\alpha}$ from the Day-15 guinea-pig uterus superfused *in vitro* (Poyser, 1985a, 1985b). However, trifluoperazine and W-7 do inhibit the basal output of $PGF_{2\alpha}$ from the Day-15 guinea-pig endometrium in culture for several days (Riley and Poyser, 1987a). This tendency and the previous results from superfusion experiments (Poyser, 1984b, 1985a, 1985b) indicate that, once activated *in vivo*, PLA_2 is capable of stimulating PG synthesis *in vitro* for several hours but, over a longer time period, continued stimulation of PLA_2 requires the influx of extracellular calcium to refill intracellular stores. The renewed intracellular calcium requires calmodulin to stimulate PLA_2 and continue PG synthesis. The decrease in the activity of PLA_2 *in vitro* over several days may be due to the loss of a protein normally stimulated by progesterone and oestradiol *in vivo* which mobilises intracellular calcium.

The sodium fluoride-induced stimulation of PG output by the superfused Day-7 guinea-pig uterus was unaffected by the PLC inhibitor, neomycin, at a concentration which markedly inhibits PLC activity in guinea-pig inner ear tissues and brain tissues (Orsukalova *et al.*, 1976: Schacht, 1976). This finding indicates that sodium fluoride does not stimulate PG synthesis by activating PLC with the resultant release of intracellular calcium by IP_3 and activation of PLA_2. This result agrees with the report that neomycin prevents IP_3 formation but not thromboxane synthesis in human platelets (Fuse and Tai, 1987). In addition, a delayed contractile response to sodium fluoride, compared with the rapid onset of receptor mediated breakdown of PIP_2, was seen in the

guinea-pig myometrium (Marc, Leiber and Harbon, 1988), which is similar to the delayed pattern of stimulation of PG synthesis seen in the guinea-pig uterus with sodium fluoride. The effect of sodium fluoride on the guinea-pig myometrium was also insensitive to pertussis toxin (Marc et al., 1988).

Overall the studies in this section suggest that the increase in endometrial $PGF_{2\alpha}$ production after Day-11 of the cycle in the guinea-pig may involve a fluoride-sensitive, toxin-insensitive G-protein. However, the finding that prolonged treatment with 10mM sodium fluoride inhibits endometrial protein synthesis and $PGF_{2\alpha}$ production (like other inhibitors of protein synthesis) suggests that the synthesis of endometrial proteins may be involved in the stimulation of endometrial $PGF_{2\alpha}$ production. Consequently, in the next series of experiments, the effects of endometrial proteins produced by the guinea-pig endometrium on endometrial $PGF_{2\alpha}$ synthesis have been investigated.

3:3 INVESTIGATIONS INTO THE CHARACTER AND ACTIVITIES OF PROTEINS SECRETED BY DAY-15 GUINEA-PIG ENDOMETRIUM

3:3:a Isolation of proteins from the culture medium of Day-15 guinea-pig endometrium

Introduction

Oestradiol acting on a progesterone-primed uterus is responsible for the increase in uterine $PGF_{2\alpha}$ output which occurs at the end of the oestrous cycle in guinea-pigs (Blatchley and Poyser, 1974; Poyser, 1983b). The mechanism by which steroids such as progesterone and oestradiol function is determined by binding to a nuclear receptor and regulating the transcription of mRNA which encodes the information required for the synthesis of new proteins.

Since many of the actions of oestradiol on the uterus are mediated through increased protein synthesis (Brenner and West, 1975), several studies have been carried out to determine the effect of protein synthesis inhibitors on guinea-pig uterine PG production and luteal function. Intrauterine, but not systemic, administration of actinomycin D on Day 10 prolonged both luteal function, as measured by plasma progesterone levels, and oestrous cycle length in guinea-pigs (Poyser, 1979). In addition, $PGF_{2\alpha}$ production by uterine homogenates on Day 15 of the cycle was lower in guinea-pigs which had received actinomycin D *in vivo* than in untreated guinea-pigs. Further studies showed that intrauterine administration of actinomycin D on Day 10 reduced $PGF_{2\alpha}$ output from the Day-15 uterus superfused *in vitro* by 80-85%; the PGE_2 output was reduced by 50% and the 6-keto-$PGF_{1\alpha}$ output was unaffected (Poyser and Riley, 1987). Thus actinomycin D selectively inhibited $PGF_{2\alpha}$ and PGE_2 synthesis and release. It was proposed that oestradiol

induces the synthesis of a protein ("lipostimulin") which, acting on a progesterone-primed uterus, "switches on" endometrial $PGF_{2\alpha}$ synthesis and release by raising the endometrial intracellular free calcium concentration and thereby activating endometrial PLA_2 (Poyser, 1984a).

Further evidence in support of this theory was provided by the demonstration that the protein synthesis inhibitors actinomycin D, cycloheximide and puromycin could reduce the outputs of $PGF_{2\alpha}$ and PGE_2 from Day-7 and Day-15 guinea-pig endometrium in culture and, at the same time, inhibit the synthesis of secreted and cellular proteins by the same tissues (Riley and Poyser, 1989). Thus, this experiment linked PG production to protein synthesis. In addition, the synthesis of secreted proteins, but not of cellular proteins, was found to be greater by Day-15 than by Day-7 endometrium in culture, implying that a secreted protein may be responsible for the increase in endometrial $PGF_{2\alpha}$ output at the end of the cycle.

Uterine protein secretion varies throughout the cycle along with changes in oestrogen and progesterone levels in plasma in a number of species, including the pig (Murray, Bazer, Wallace and Warnick, 1972), cow (Dixon and Gibbons, 1979), mouse (Fishel, 1979), horse (Zavy, Bazer, Sharp and Wilcox, 1979), cat (Murray, Jaffe, Buhi and Verhage, 1983) and human (Bell, Patel, Kirwan and Drife, 1986; Heffner, Iddenden and Lyttle, 1986). Few of these proteins have an identified function as yet.

The experiments reported here have sought to purify and identify proteins secreted by the Day-15 guinea-pig endometrium which might be involved in the stimulation of uterine $PGF_{2\alpha}$ output, which occurs towards the end of the cycle and is dependent upon new protein synthesis induced by oestradiol acting on a progesterone-

primed uterus. The effects of the proteins isolated were tested on the output of PGs from Day-7 guinea-pig endometrium in culture and on the activity of PLA_2, in order to determine if a particular protein produced by the endometrium is responsible for the increase in endometrial $PGF_{2\alpha}$ synthesis at the end of the cycle.

Methods

The uteri were removed from thirty-two Day-15 guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 8 petri dishes containing 30-50mg wet weight (6-10mg dry weight) of endometrium were prepared from each uterus. The dishes were incubated at 37°C for 24h (see Section 2:5). The medium from each dish was removed and pooled before undergoing dialysis and desalting as the first steps of purification (see Section 2:10). The tissue from each dish was amalgamated in a preweighed container and dried by placing in an oven at 37°C for 24h. The container was then reweighed and the amount of dried endometrium was calculated.

After dialysis and desalting, the medium was lyophylized and the amount of protein present was determined by Lowry assay (see Section 2:10). The molecular weights of the proteins present were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)(see Section 2:8).

Results

A total of 2.04g of dried endometrial tissue was obtained from 32 guinea-pigs (approximately 60mg/animal). After dialysis, desalting and lyophylization the culture medium obtained from 32 guinea-pigs

yielded 356.9mg of dried material (approximately 11mg/animal). The dried material was found to be 80-90% protein by Lowry assay, resulting in a weight of dried protein of between 285.5-321.2mg.

The molecular weight profile of the unpurified endometrial secretory protein (uESP) is shown in Figure 25. A heavily stained band was found at 68.3kDa. The 68.3kDa protein co-migrated with the molecular weight marker for bovine serum albumin (67kDa). Other protein bands were observed at 97.4kDa, 94.6kDa, 74.3kDa with light, diffuse staining also occurring around 15.6kDa.

Conclusions

Approximately 14-16% of the dried weight of endometrial tissue was secreted as protein during 24h of culture. However, the large band with a relative mobility similar to that of the molecular weight marker for bovine serum albumin (67kDa), observed on the SDS PAGE profile of unpurified endometrial secretory protein (uESP) from the guinea-pig, suggests that some of the protein recovered may have originated in serum. Thus, the proteins purified from the culture medium from Day-15 guinea-pig endometrium consisted of proteins from serum which may have "leached" from the cultured tissue but have not been synthesised by it, as well as proteins synthesised and secreted by the endometrium.

In order to remove the serum albumin contamination, it was decided to purify the guinea-pig endometrial secretory proteins by affinity chromatography on Blue Sepharose.

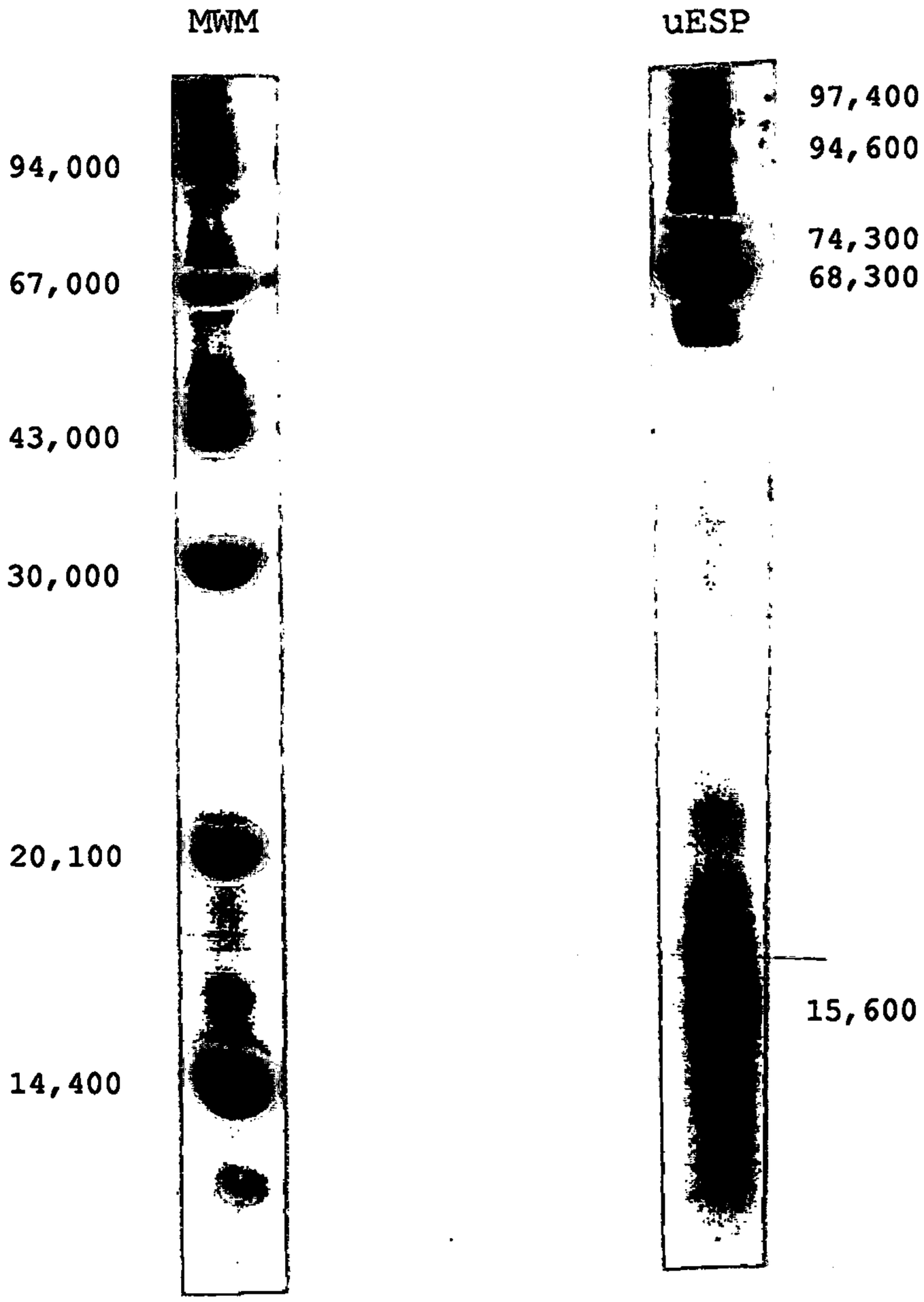


Fig. 25. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profile of unpurified endometrial secretory proteins (uESP) from the Day-15 guinea-pig. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of uESP were run on the gel.

3:3:b—— Purification of proteins secreted by Day-15 guinea-pig endometrium by affinity chromatography

Introduction

Analysis of the molecular weights of proteins purified from the culture medium from Day-15 guinea-pig endometrium by SDS PAGE revealed the presence of large amounts of serum albumin. In order to remove the albumin contamination, the endometrial secretory proteins were subjected to purification by affinity chromatography on Blue Sepharose CL-6B. Blue Sepharose CL-6B consists of the dye Cibacron Blue F3 G-A covalently attached to Sepharose CL-6B. Cibacron Blue F3 G-A binds a wide variety of enzymes and other proteins including albumin (Travis et al., 1976), and has been shown to remove albumin from human uterine flushings (MacLaughlin and Richardson, 1983).

Methods

The lyophylized protein obtained from Day-15 guinea-pig endometrium was run in batches at a concentration of not more than 50mg/ml (higher protein concentrations result in an increase in the viscosity of the sample resulting in an uneven flow of protein through the column) on a Blue Sepharose affinity column (see Section 2:10:(i)). Proteins were separated into two fractions: (i) the proteins which eluted directly from the column (i.e. the proteins which had no affinity for Blue Sepharose, henceforth known as PBS) and (ii) the proteins which were retained on the column and were eluted with a high salt buffer (i.e. the proteins which bound to Blue Sepharose, henceforth known as ROBS). Each protein fraction was dialysed, desalted and lyophylized (see Section 2:10) before being weighed. The molecular weights of the proteins present in each of

the PBS and ROBS fractions were determined by SDS PAGE (see Section 2:8).

Results

A total of 319.6mg of protein was run on Blue Sepharose of which 194.2mg of protein were recovered (61%). Of the 194.2mg of protein recovered, 189.5mg (97.6%) of protein were present in the PBS fraction and 4.7mg (2.4%) of protein were present in the ROBS fraction.

The molecular weight profiles of the PBS and ROBS fractions are shown in Figure 26. In the profile corresponding to the proteins which were retained on the column until elution with high salt buffer (ROBS), the protein band at 74.3kDa is missing indicating a lack of affinity of Blue Sepharose for this protein. There is also a heavily stained band at 68.3kDa corresponding to bovine serum albumin (67kDa) in this profile indicating that the Blue Sepharose has a high affinity for albumin. However, the profile corresponding to the proteins which eluted directly from the column and were not bound to Blue Sepharose (PBS) also has a band, although less heavily stained, with a relative mobility similar to bovine serum albumin (67kDa).

Conclusions

The protein band at 74.3kDa which does not bind to Blue Sepharose may be transferrin which has a similar molecular weight (76.5kDa). Indeed, Blue Sepharose has been used to ultra-purify commercially produced transferrin, which usually contains albumin as a contaminant, as it has no affinity for transferrin (Pharmacia AB, Uppsala, Sweden). The inability of Blue Sepharose to entirely remove

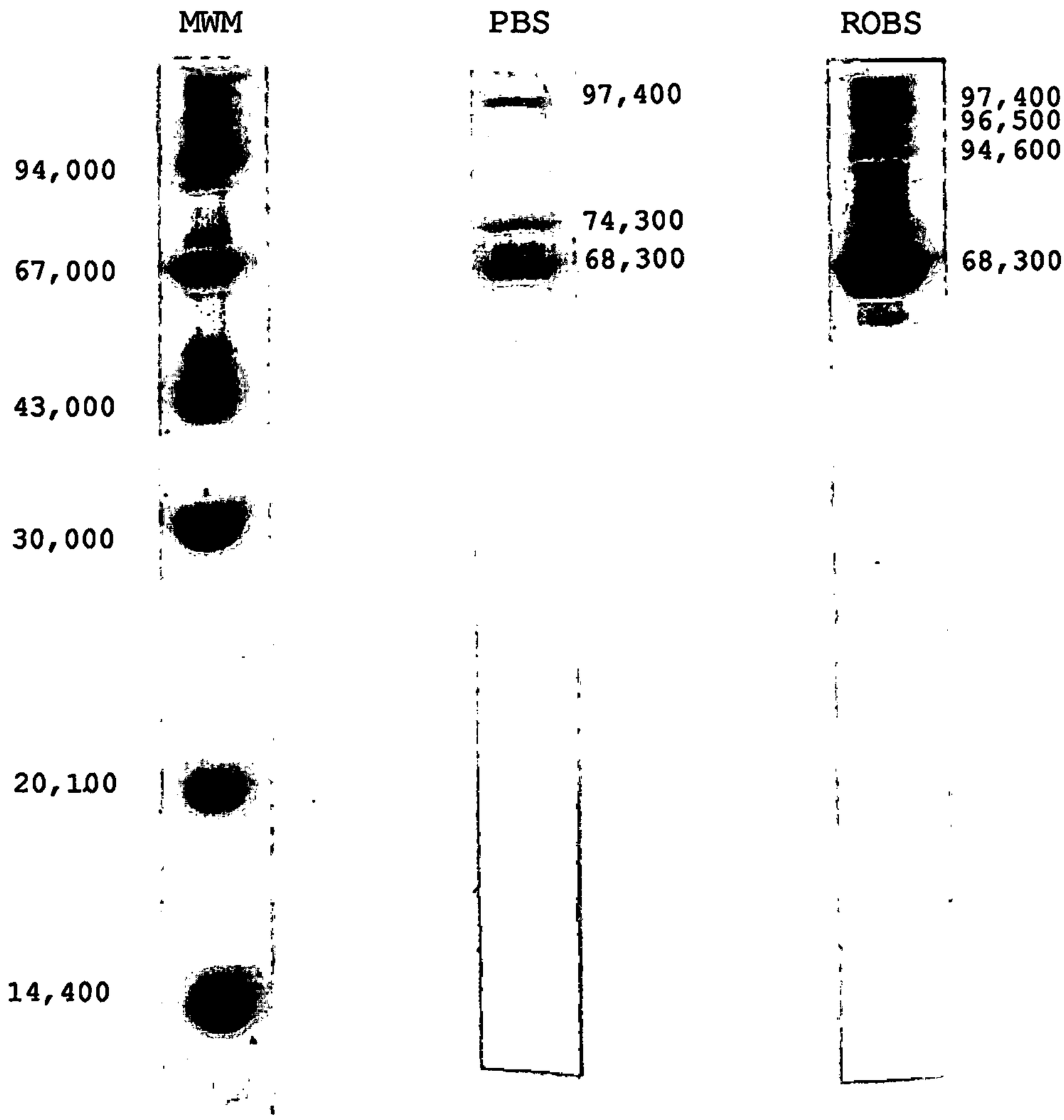


Fig. 26. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of endometrial secretory proteins from the Day-15 guinea-pig (i) which had no affinity for Blue Sepharose (PBS) and (ii) which were retained on Blue Sepharose (ROBS). The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

the albumin band from the sample which passed directly through the column (PBS) may be due to the fact that albumin is one of the group of proteins whose interaction with the Cibacron Blue dye is nonspecific (i.e. it is based upon electrostatic and/or hydrophobic interactions). The composition of the buffer used may therefore not have been at the optimal pH or concentration to allow the maximum binding of albumin to the column. In addition, the SDS PAGE profile of the ROBS fraction exhibited several high molecular weight protein bands which were also present on the SDS PAGE profile of unpurified endometrial secretory proteins (uESP; Figure 25). This implies that either these proteins also had an affinity for Blue Sepharose or that nonspecific binding of proteins to the column occurred. As only 10μg of PBS proteins were run on the PAGE gel and a couple of major proteins masked anything else which may have been there in smaller quantities, further purification techniques were carried out.

3:3:c Purification of proteins secreted by Day-15 guinea-pig endometrium by ion-exchange chromatography

Introduction

In order to identify and purify further the proteins obtained from the culture medium from Day-15 guinea-pig endometrium, the PBS fraction was subjected to separation by ion-exchange chromatography. Ion-exchange is a high resolution chromatographic technique with separation being achieved on the basis on the charges carried by the protein. The anionic exchanger diethylaminoethyl (DEAE) linked to Sepharose CL-6B was used in conjunction with the Pharmacia Gradient Mixer GM-1 in order to obtain an elution of proteins with an ionic gradient. At low ionic strengths, competition for charged groups on the ion-exchanger is at a minimum and proteins are bound strongly. Increasing the ionic strength increases competition and reduces the interaction between the ion exchanger and the sample proteins, resulting in their elution. Ion-exchange chromatography is therefore able to resolve extremely complex mixtures of proteins, as proteins which have closely similar molecular weights may be separated on the basis of very small differences in charge.

Methods

The lyophylized protein fraction which had eluted directly from the Blue Sepharose column (i.e. had no affinity for Blue Sepharose; PBS) was separated on DEAE-Sepharose CL-6B in 3 batches by dissolving approximately 50mg of protein in 2ml of DEAE Sample buffer. The protein was applied to the column and was eluted with an ionic gradient of 0-0.5M NaCl (see Section 2:10:(ii)). The proteins were collected on a Pharmacia Fraction Collector Frac-100 in 5ml

volumes and were separated into 7 fractions on the basis of the peaks of absorbance observed on the chart recording of the elution profile. Each fraction was dialysed, desalted and lyophylized before being weighed. The molecular weights of the proteins present in each fraction were determined by SDS PAGE (see Section 2:8).

Results

Of the 141.3mg of dried endometrial protein applied to the ion exchange column, 109mg (77%) were recovered. The elution profile of the Day-15 guinea-pig endometrial secretory protein fraction PBS on DEAE-Sepharose CL-6B is shown in Figure 27. The protein was separated into 7 fractions henceforth known as F1-F7. The elution of F1, F2, F3, F4, F5, F6 and F7 occurred at NaCl concentrations of 0.1M, 0.125M, 0.175M, 0.2M, 0.225M, 0.3M and 0.375M, respectively. The total amount of protein found in each of the 7 fractions, after amalgamation of each of the fractions from each run, was 2.6mg, 5.3mg, 11.1mg, 21.0mg, 36.5mg, 24.0mg and 8.5mg in F1, F2, F3, F4, F5, F6 and F7, respectively.

The molecular weight profiles of the proteins in F1-F7 analysed by SDS PAGE are shown in Figure 28. F1 contained lightly stained protein bands at 97.4kDa, 68.3kDa and 19.6kDa (Figure 28). F2 contained a heavily-stained high molecular weight protein at 97.4kDa with further bands at 96.4kDa, 94.6kDa, 74.3kDa, 68.3kDa, 57.8kDa and 31.3kDa. Wide bands of protein staining were also found in the low molecular weight region at 19.6kDa and 13.2kDa (Figure 28). F3 contained proteins at 97.4kDa, 95.7kDa, 74.3kDa and 72.1kDa as well as a heavily-stained band at 63.2kDa (Figure 28). Proteins were found in F4 at 97.4kDa, 96.4kDa, 95.7kDa and 94.6kDa with large, heavily-stained bands of protein also present at 74.3kDa and 68.3kDa

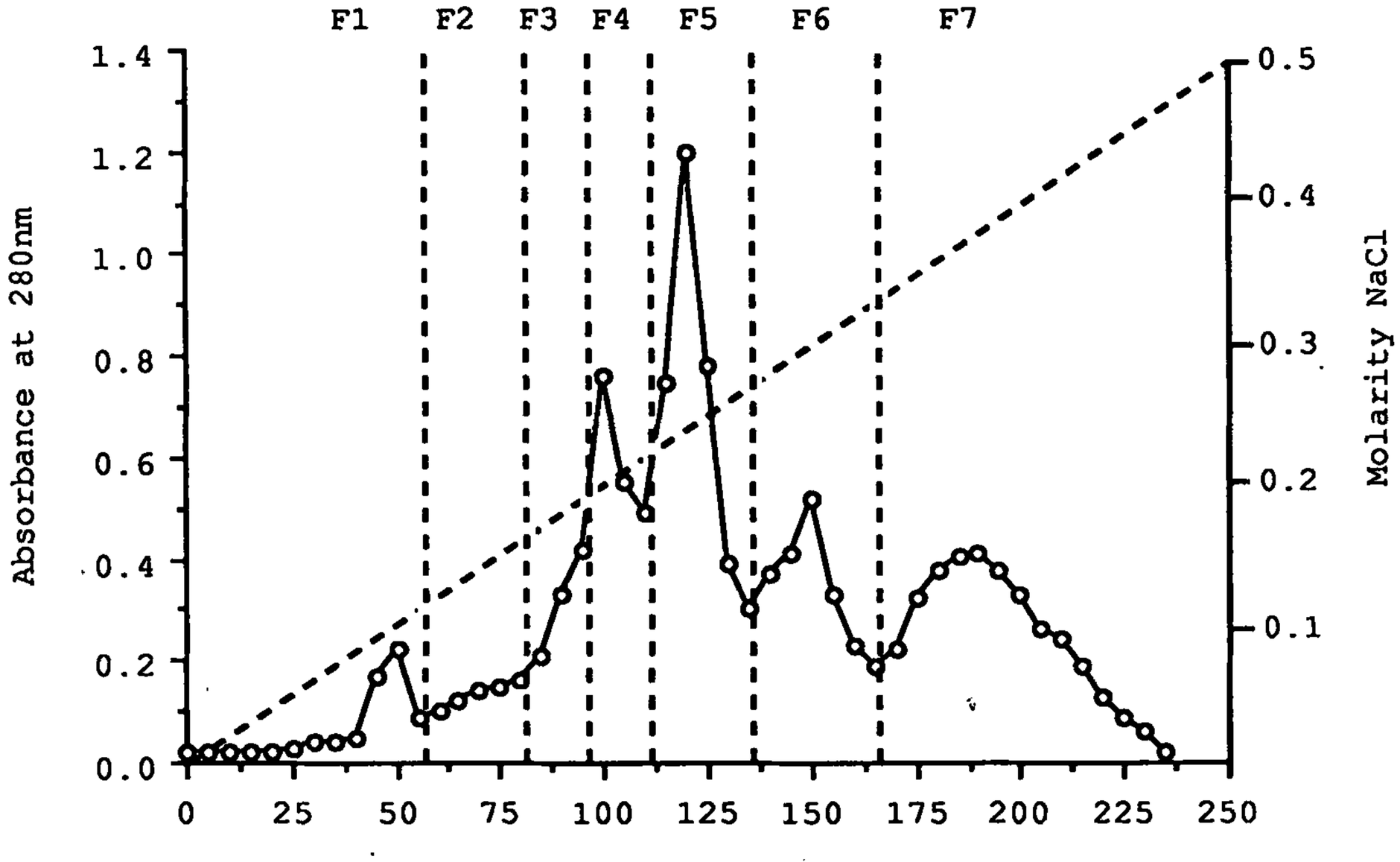


Fig. 27. The elution profile of guinea-pig endometrial secretory proteins, which were not retained on Blue Sepharose (PBS; 33.45mg/ml), from an ion-exchange column (DEAE Sepharose CL-6B) with an ionic gradient of 0-0.5M NaCl. The proteins were collected on a Fraction Collector in 5ml volumes and separated into 7 fractions (F1-F7) on the basis of the peaks of absorbance observed on the elution profile.

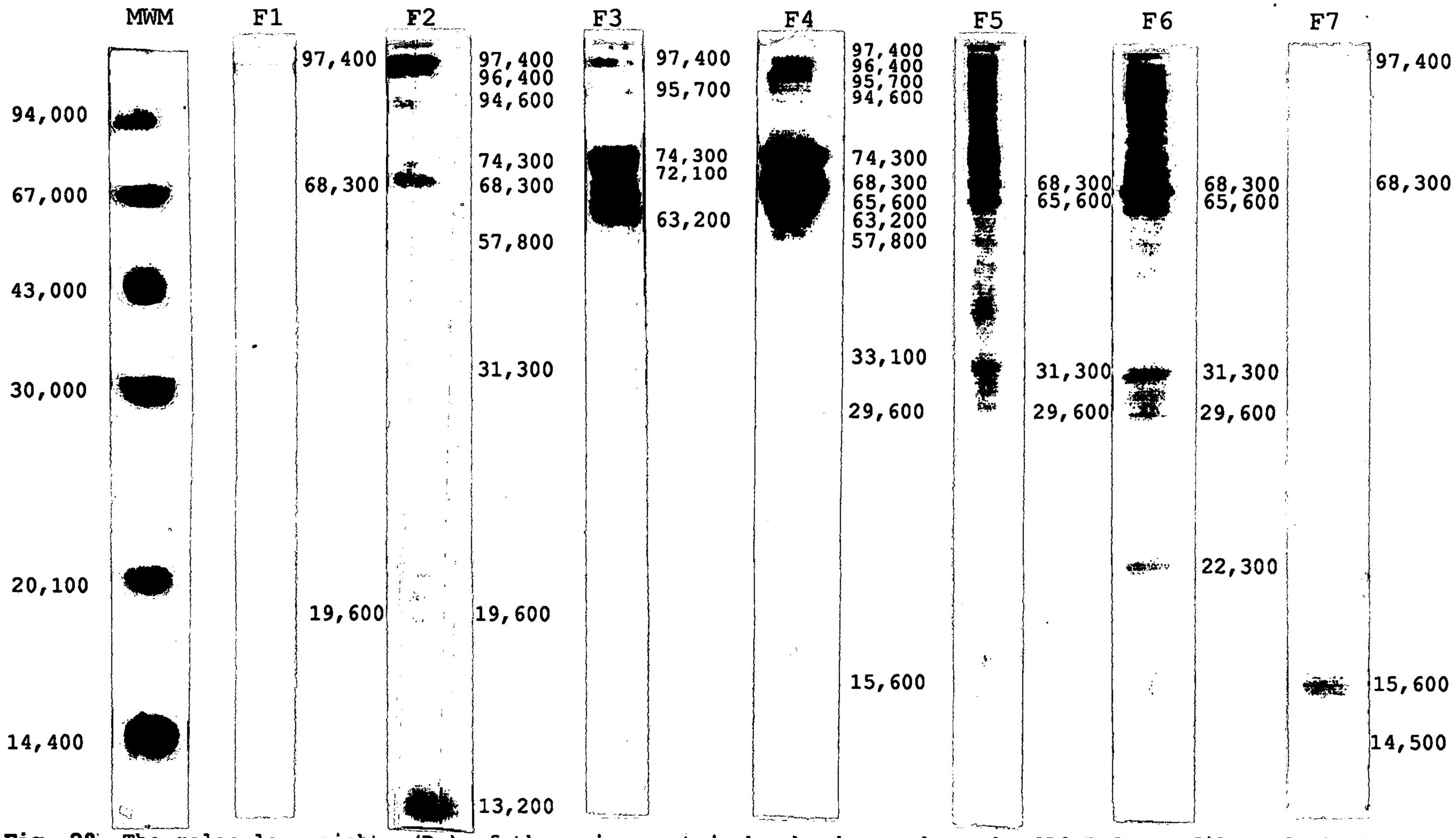


Fig. 28. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of F1-F7 of guinea-pig endometrial secretory proteins from the PBS fraction separated by ion-exchange chromatography. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

continuing down to 57.8kDa. A lightly stained band was also seen at 15.6kDa (Figure 28). F5 and F6 exhibited similar protein profiles to each other, with stained bands ranging the length of the gel (approximately 10,000-100,000 Da). However F6 contained a protein band at 22.3kDa which was not present in F5 (Figure 28). The majority of the protein in F7 was found in the low molecular weight region with bands at 15.6kDa and 14.5kDa (Figure 28).

Conclusions

Separation of endometrial proteins on the basis of the charge carried by each protein resulted in the successful resolution of the proteins in the PBS fraction into 7 protein peaks which eluted over an ionic concentration range of 0-0.5M NaCl. Several proteins below 65kDa, which were not prominent bands on the PAGE profile of unpurified endometrial secretory protein (uESP; Figure 25), were found in the fractions after ion-exchange chromatography. The low molecular weight fractions were found predominantly in F1, F2 and F7, while the majority of the serum protein contaminants, albumin and transferrin, were resolved into F3 and F4. F3 and F4 were eluted at an ionic concentration of 0.15-0.215M NaCl. Albumin has been shown to elute at 0.2M NaCl when undergoing separation from haemoglobin on DEAE Sephadex A-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

3:3:d— Purification of proteins secreted by Day-15 guinea-pig endometrium by gel filtration chromatography

Introduction

A range of proteins with a wide variation in molecular weight was found in each fraction of guinea-pig endometrial protein (PBS) separated by ion-exchange chromatography (F1-F7). Therefore, it was decided to further purify each fraction by gel filtration chromatography on Sephadex G-75 Superfine (SF). Gel filtration separates proteins on the basis of molecular weight with proteins being eluted from the column in decreasing order of molecular size. Small proteins enter pores in the gel beads which retard their progression through the column whereas large proteins are excluded from the gel pores and pass more quickly through the column. Sephadex G-75 SF has a fractionation range of 3,000-70,000 and was used to separate endometrial proteins into a high molecular weight fraction (>70,000), an intermediate molecular weight fraction (70,000-60,000) and a low molecular weight fraction (<60,000).

Methods

Fractions F3-F7 obtained from ion-exchange chromatography of PBS proteins (Section 3:3:c), were further purified by gel filtration chromatography on Sephadex G-75 SF. There were insufficient amounts of F1 and F2 remaining after SDS PAGE analysis to allow further purification while retaining enough protein for testing later on.

Each fraction was dissolved in 2ml of Sample Buffer and applied to the Sephadex G-75 SF column as described in Section 2:10:(iii). The eluted proteins were collected in 2ml fractions on a Fraction Collector and separated into high, intermediate and low molecular

weight fractions according to the peaks of absorbance observed on the chart recording of the elution profile. The molecular weight of each protein peak was calculated from the elution volume of the peak using the calibration curve for the Sephadex G-75 SF column (see Section 2:10:(iii), Figure 13). After dialysis, desalting and lyophylization each fraction was weighed and the molecular weights of the proteins present were determined by SDS PAGE (see Section 2:8).

Results

The elution profiles of F3-F7 from the Sephadex G-75 SF column are shown in Figure 29. The amount of protein in each fraction is shown in Table 8.

The molecular weight profiles of the high (1), intermediate (2) and low (3) molecular weight fractions of F3-F7 on SDS PAGE are shown in Figures 30-34. F3:1 exhibited proteins at 97.4, 94.6kDa, 74.3kDa and 68.3kDa (Figure 30). F3:2 exhibited proteins at 97.4kDa and 94.6kDa with large heavily stained bands at 74.3kDa and 68.3kDa. Staining continued down to a band at 38.2kDa (Figure 30). F3:3 exhibited proteins at 30.1kDa, 29.6kDa, 15.6kDa and 13.5kDa (Figure 30). F4:1 contained proteins ranging from 96.5-45.6kDa (Figure 31). F4:2 contained proteins at 96.5kDa and 94.6kDa with heavily stained bands at 74.3kDa and 68.3kDa continuing down to 63.2kDa (Figure 31). F4:3 contained proteins ranging from 74.3-13.5kDa (Figure 31). F5:1 contained proteins in the range 97.4-40.2kDa (Figure 32). F5:2 contained proteins ranging from 97.4-15.6kDa with a very large band at 68.3kDa (Figure 32). F5:3 contained proteins ranging from 68.3-13.5kDa (Figure 32). F6:1 contained proteins ranging from 97.4-13.5kDa (Figure 33). F6:2 contained proteins ranging from

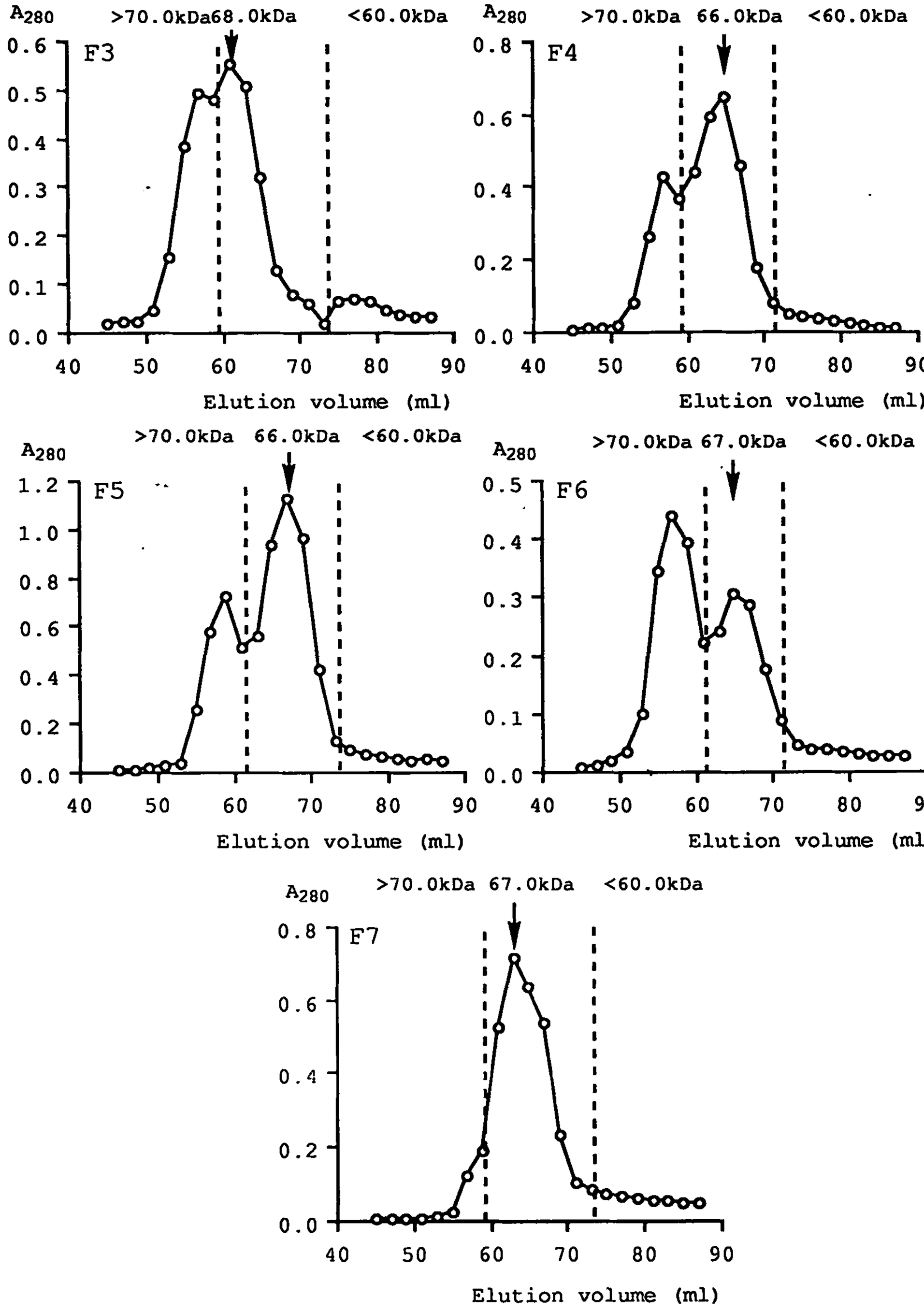


Fig. 29. The elution profiles of F3 (4.85mg/ml), F4 (7.85mg/ml), F5 (15.75mg/ml), F6 (10.45mg/ml) and F7 (3.45mg/ml) from a Sephadex G-75 SF gel filtration column. The proteins were collected on a Fraction Collector in 2ml volumes and separated into fractions on the basis of the peaks of absorbance observed on each elution profile. A_{280} = absorbance at 280nm.

Table 8. The amount of protein recovered in the high (1), intermediate (2) and low (3) molecular weight fractions of the Day-15 guinea-pig endometrial secretory proteins F3-F7 after separation on Sephadex G-75 SF.

	Amount of protein (mg) in each fraction after separation on Sephadex G-75 SF		
Fraction from ion-exchange	1	2	3
F3	0.8	1.9	2.0
F4	1.7	6.6	0.3
F5	2.4	16.0	2.1
F6	3.8	9.4	2.2
F7	0.5	3.0	1.8

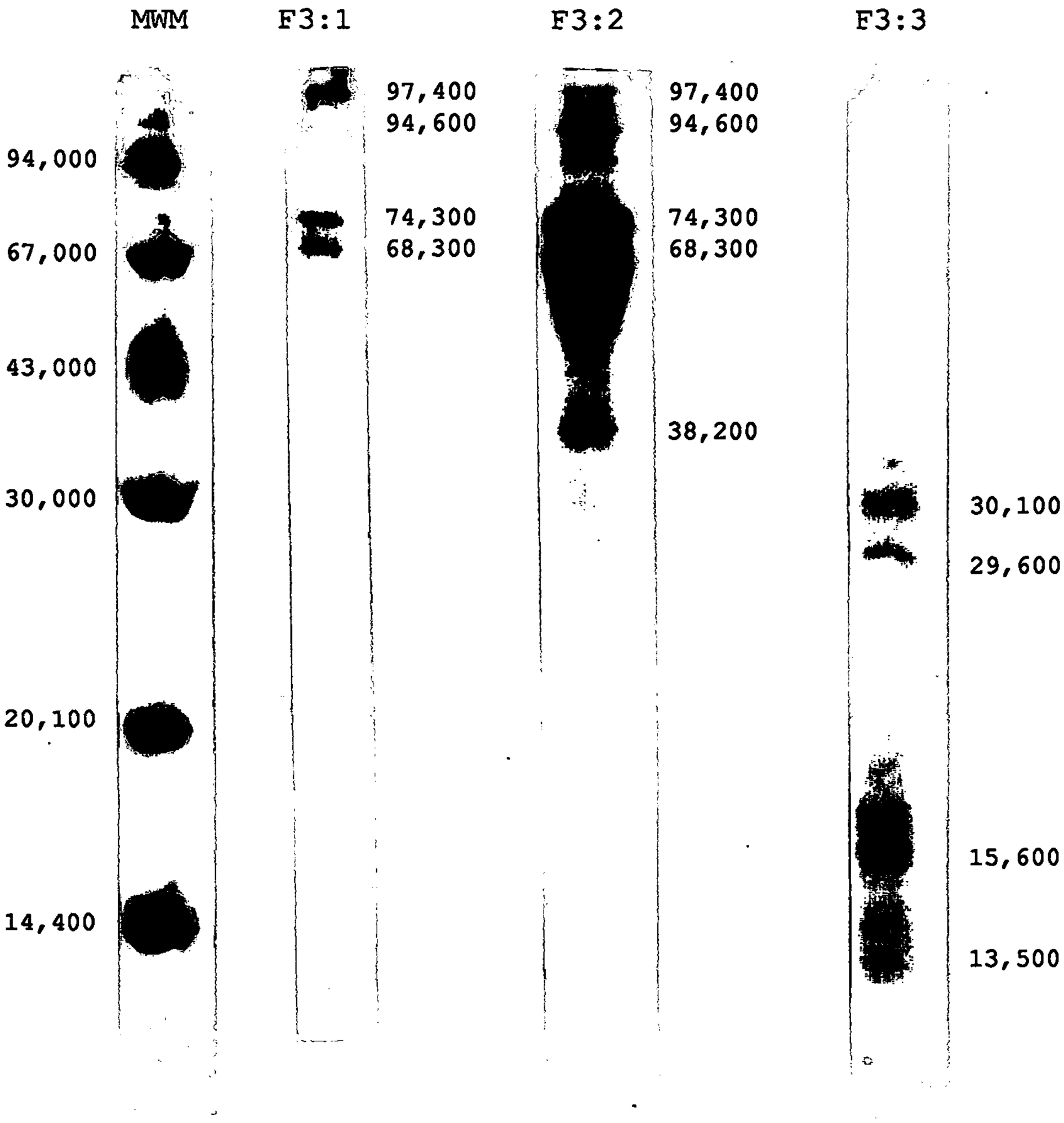


Fig. 30. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig endometrial secretory proteins purified from the PBS fraction, F3:1, F3:2 and F3:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10μg of each protein fraction were run on the gel.

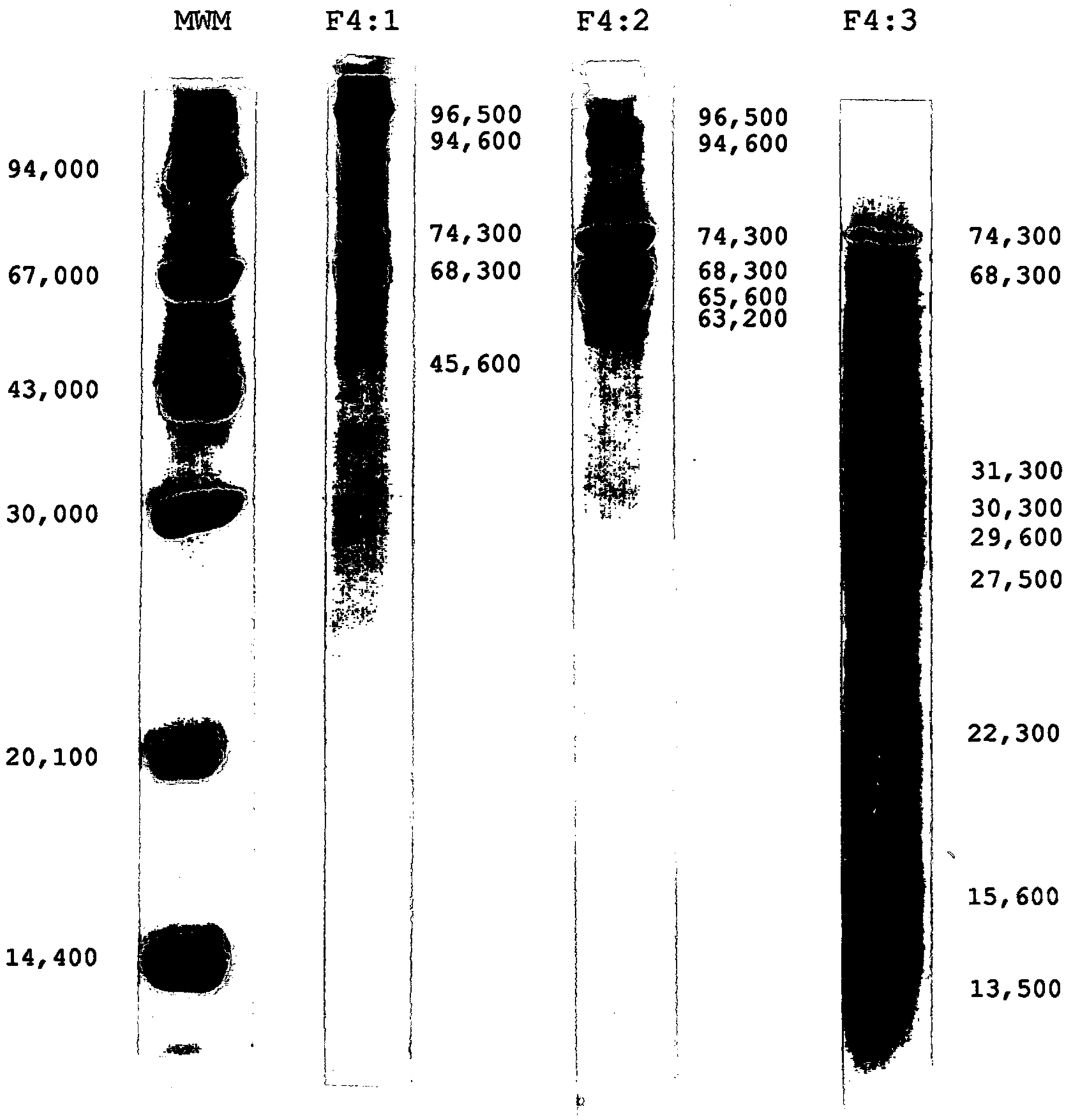


Fig. 31. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig endometrial secretory proteins purified from the PBS fraction, F4:1, F4:2 and F4:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

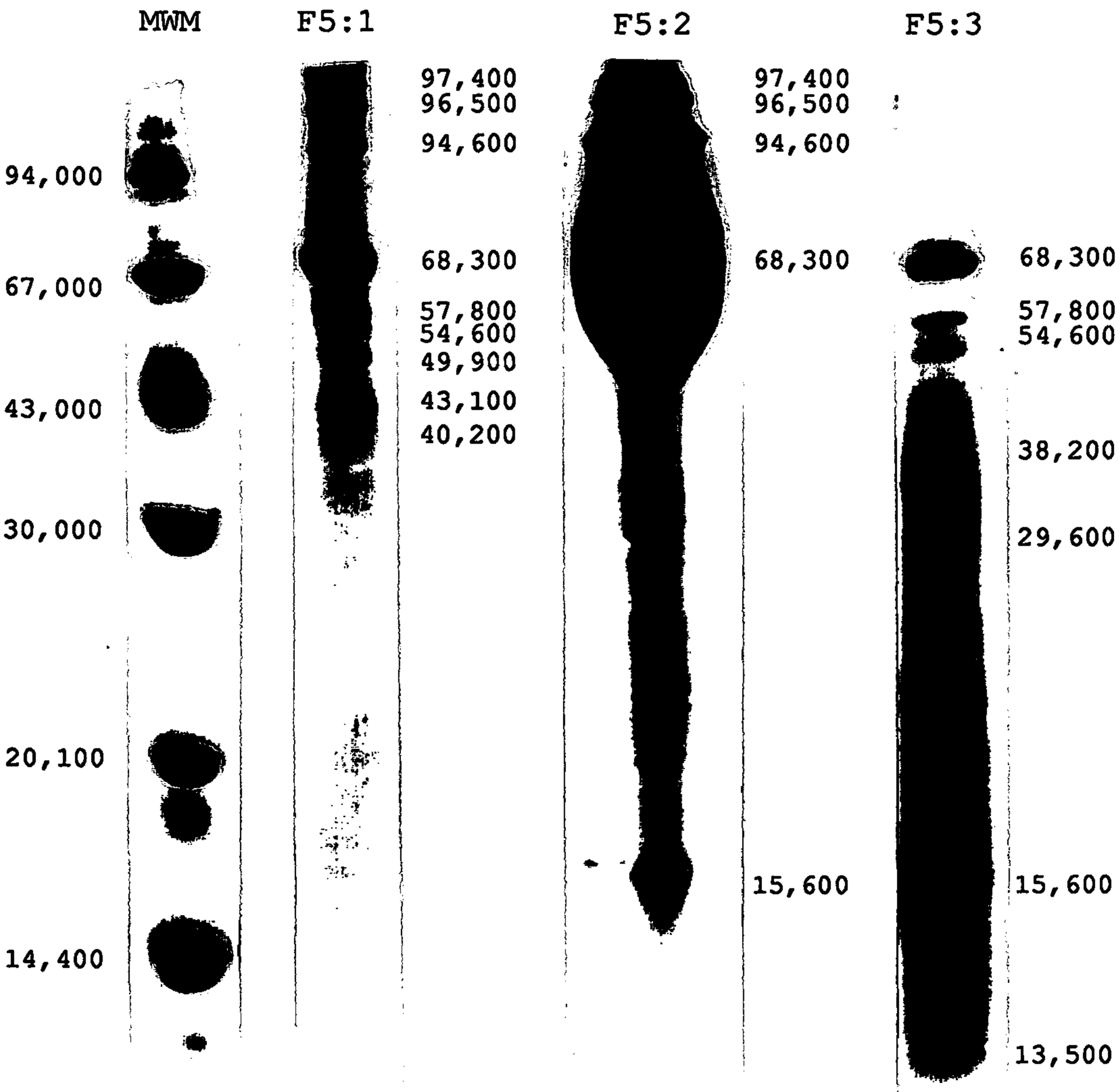


Fig. 32. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig endometrial secretory proteins purified from the PBS fraction, F5:1, F5:2 and F5:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

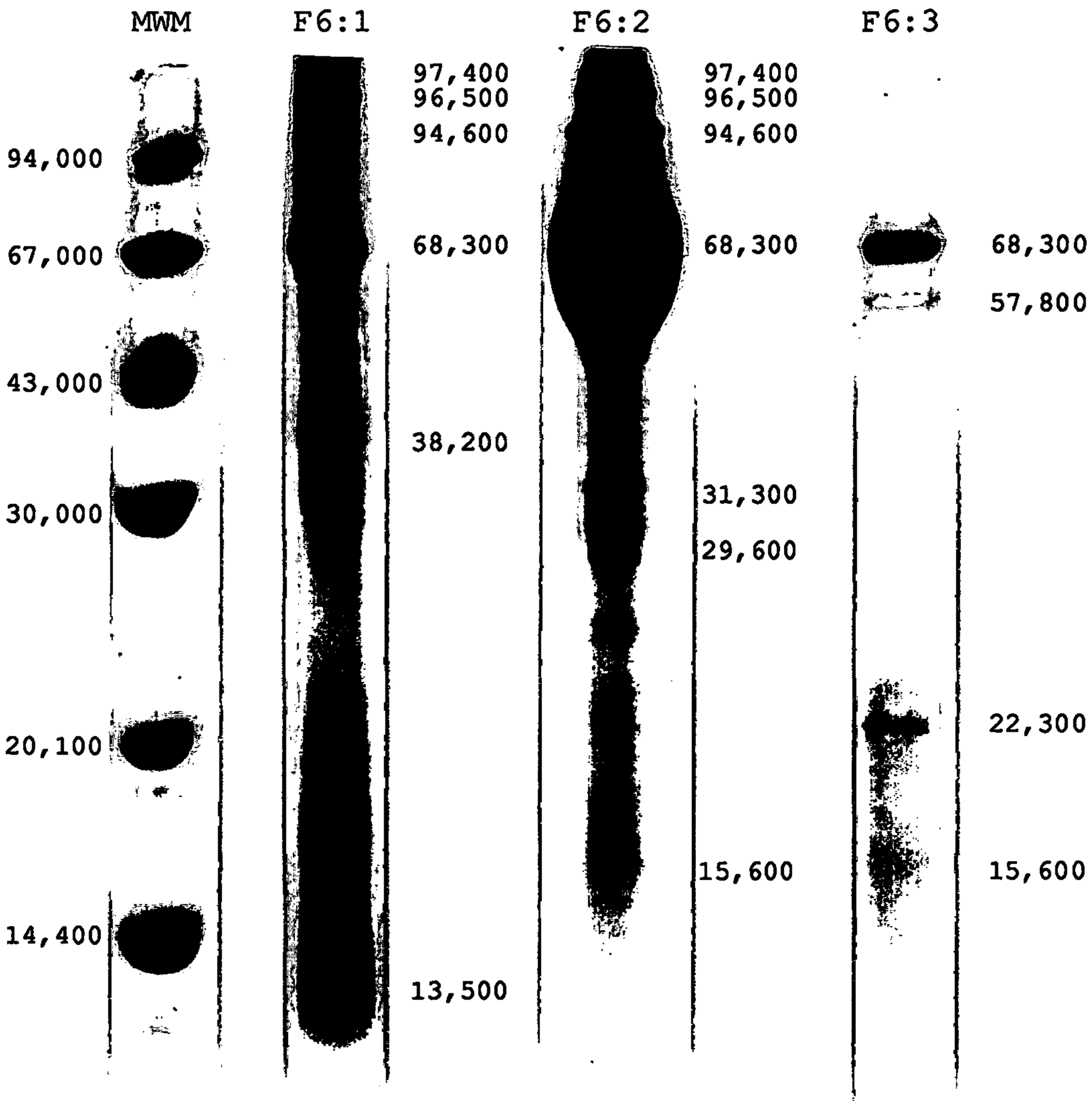


Fig. 33. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig endometrial secretory proteins purified from the PBS fraction, F6:1, F6:2 and F6:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

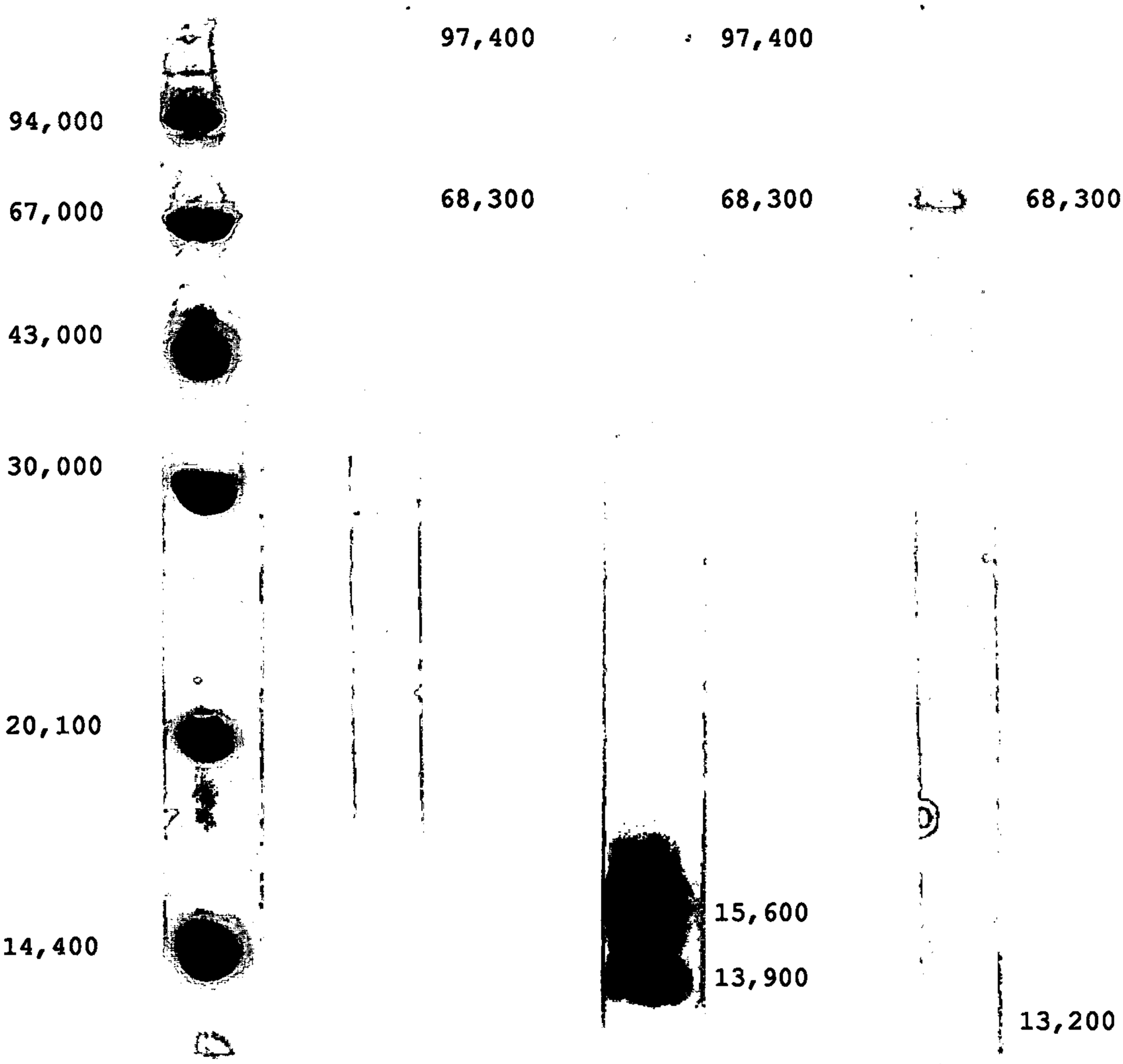


Fig. 34. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig endometrial secretory proteins purified from the PBS fraction, F7:1, F7:2 and F7:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 5µg of each protein fraction were run on the gel.

97.4-15.6kDa with a very large band at 68.3kDa (Figure 33). F6:3 contained proteins at 68.3kDa and 57.8kDa with staining increasing from approximately 30.0kDa down to 15.6kDa (Figure 33). Only lightly stained proteins at 97.4kDa and 68.3kDa were observed in F7:1 (Figure 34). Proteins at 97.4kDa and 68.3kDa were also faintly observed in F7:2 with heavily stained bands present at 15.6kDa and 13.9kDa (Figure 34). Proteins at 68.3kDa and 13.2kDa were found in F7:3 (Figure 33).

Conclusions

Separation of fractions F3-F7 (obtained after ion-exchange chromatography of endometrial proteins not retained on Blue Sepharose; PBS) by gel filtration chromatography led to the successful resolution of each fraction into high (> 70,000 Da), intermediate (60,000-70,000 Da) and low (< 60,000 Da) molecular weight fractions as determined by SDS PAGE analysis. The presence of low molecular weight proteins on the SDS PAGE profiles of some of the intermediate and high molecular weight fractions may be due to the instability of some of the proteins to the presence of the denaturing agent, SDS, resulting in formation of lower molecular weight proteins. The presence of high molecular weight proteins on the SDS PAGE profiles of some of the intermediate and low molecular weight fractions may be due to the large quantities of some of these proteins resulting in nonspecific binding to the column and their being eluted along with other lower molecular weight fractions. The majority of the albumin from each fraction was found in the intermediate (2) molecular weight fraction as would be expected. It was decided, as there were very small amounts of proteins remaining in some of the fractions after undergoing this series of

chromatographic techniques, that no further purification of the Day-15 guinea-pig endometrial proteins would be carried out and the effects of each fraction on the output of PGs from the Day-7 guinea-pig endometrium in culture were tested.

3:3:e The effects of Day-15 guinea-pig endometrial secretory proteins on the output of prostaglandins from Day-7 guinea-pig endometrium in culture

Introduction

To determine whether any of the protein fractions purified from the culture medium of Day-15 guinea-pig endometrium contained the protein mediator responsible for the increase in $PGF_{2\alpha}$ synthesis in the guinea-pig uterus at the end of the cycle, the effect of each of the protein fractions on the output of PGs from Day-7 guinea-pig endometrium in culture was examined.

Day-7 is a day of low uterine PG output (particularly of $PGF_{2\alpha}$) in the guinea-pig. Several compounds including sodium fluoride (see Section 3:2), A23187 (Poyser and Brydon, 1983), arachidonic acid (Poyser, 1985a), PLA_2 and PLC (Poyser, 1987a), melittin (Johnson and Poyser, 1991) and platelet-activating factor (PAF) (Norman and Poyser, unpublished observations) are capable of stimulating PG output from the Day-7 guinea-pig uterus superfused *in vitro*. To maximise the concentration of protein to which the endometrium was exposed and to ensure that the proteins had sufficient time to act, the effects of each protein fraction on PG output from the Day-7 guinea-pig endometrium was assessed in culture over a period of 12h.

Methods

As there were very small amounts of some of the protein fractions remaining after purification and molecular weight analysis by SDS PAGE, the effect on PG synthesis of every protein fraction could not be determined. Only those fractions of which there remained sufficient protein to allow a concentration of at least 20μg/ml in

each petri dish were tested.

The uteri were removed from five Day-7 guinea-pigs and, under aseptic conditions, each uterine horn was "opened" by a longitudinal incision. The endometrium was dissected away from the myometrium and cut into 1-2mm^3 pieces. 36 petri dishes each containing 5-10mg wet weight (1-2mg dry weight) of endometrium were prepared from each uterus. Pairs of dishes were treated with unpurified endometrial secretory protein (uESP; 700μg/ml), PBS protein (170μg/ml), F4 (105μg/ml), F5 (105μg/ml), F6 (25μg/ml), F3:1 (30μg/ml), F3:2 (35μg/ml), F3:3 (45μg/ml), F4:1 (25μg/ml), F4:2 (165μg/ml), F5:1 (60μg/ml), F5:2 (325μg/ml), F5:3 (40μg/ml), F6:1 (90μg/ml), F6:2 (205μg/ml), F6:3 (70μg/ml), F7:2 (20μg/ml) and no protein (control). Each treatment used the maximum concentration of protein possible consistent with the amount of protein available in order for the procedure to be carried out in duplicate over two time periods on five animals. In addition, to allow exposure of the tissue to as high a concentration of each protein as possible, the volume of culture medium in each petri dish was reduced from 4ml to 2ml by the use of 3.5cm diameter dishes. The petri dishes were incubated at 37°C for 12h and the culture medium was sampled and replaced with fresh medium containing the same protein treatments at 6h. The samples of culture medium obtained after both time periods (i.e. 6h and 12h) were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each dish into separate preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was then reweighed and the amount of dried endometrium in each dish was calculated. PG outputs were calculated per mg dry weight of endometrium.

Statistical tests

Changes in the output of PGs with time and differences between control and treated groups were analysed by Student's t test or, if the variances of the two groups were different by the variance ratio F test, by a modified t test for unequal variances.

Results

The control output of $PGF_{2\alpha}$ from Day-7 endometrium significantly ($P < 0.05$) increased and the control output of 6-keto-$PGF_{1\alpha}$ significantly ($P < 0.05$) decreased during 12h of culture (Figure 35). The control output of PGE_2 from Day-7 endometrium did not change during 12h of culture (Figure 35).

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 endometrium in culture at 6h and at 12h were significantly ($P < 0.01$) increased by treatment with unpurified endometrial secretory protein (uESP; 700μg/ml; Figure 35). The output of 6-keto-$PGF_{1\alpha}$ from Day-7 endometrium at 6h was significantly ($P < 0.05$) decreased by treatment with the high molecular weight fraction of F4 (F4:1; 25μg/ml). None of the other protein fractions tested significantly affected the output of PGs from the Day-7 endometrium at 6h or 12h of culture.

Conclusions

Unpurified endometrial secretory protein (uESP) stimulated the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 endometrium in culture at 6h and 12h. However, purification of Day-15 endometrial secretory protein by affinity, ion-exchange and gel filtration chromatography failed to isolate any particular protein which stimulated endometrial PG output. Consequently, the reason why

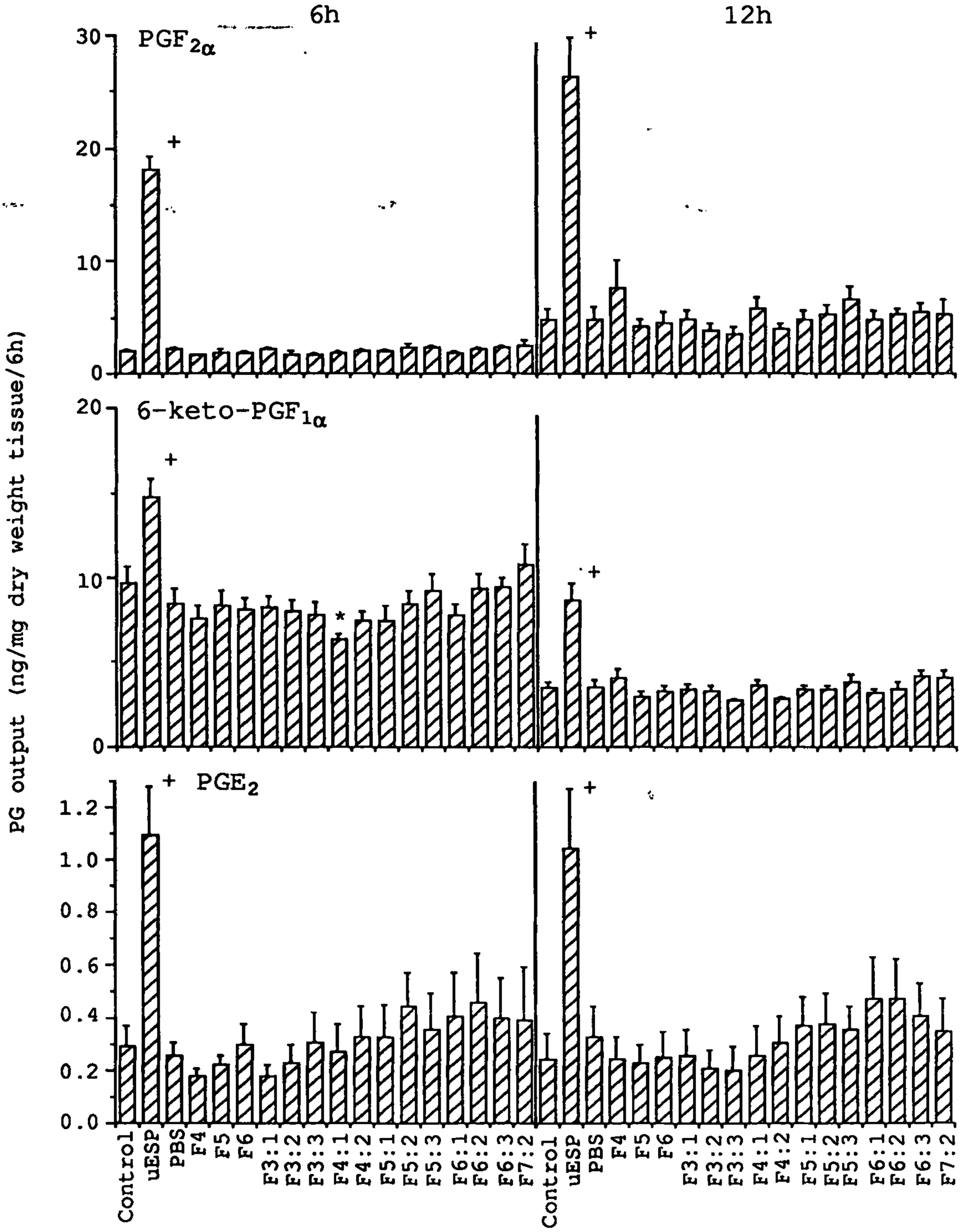


Fig. 35. The effects of uESP (700µg/ml), PBS (170µg/ml), F4 (105µg/ml), F5 (75µg/ml), F6 (25µg/ml), F3:1 (30µg/ml), F3:2 (35µg/ml), F3:3 (45µg/ml), F4:1 (25µg/ml), F4:2 (165µg/ml), F5:1 (60µg/ml), F5:2 (325µg/ml), F5:3 (40µg/ml), F6:1 (40µg/ml), F6:2 (205µg/ml), F6:3 (70µg/ml) and F7:2 (20µg/ml) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium cultured for 12h and sampled at 6h. *Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

uESP stimulated endometrial PG production - whereas none of the resolved protein fractions possessed this stimulatory effect is unclear. Many of the protein fractions tested were present in the culture medium at relatively low concentrations (down to 20μg/ml) and it may have been that these concentrations were too low for any effect on PG synthesis to be seen. Also there was insufficient protein remaining in fractions F1, F2, F3, F4:3, F7, F7:1 and F7:3 for them to be tested on Day-7 endometrium, so the stimulatory protein may have been present in these fractions, albeit in very small amounts. Alternatively, the PG stimulatory component of Day-15 unpurified endometrial secretory protein (uESP) may have been removed by affinity chromatography on Blue Sepharose. However, only 2.4% of uESP bound to the Blue Sepharose column and it was thought unlikely that this small fraction could be responsible for the PG stimulatory effect of uESP especially as the majority of protein in the ROBS fraction is serum albumin. In addition, there were no proteins in the PAGE profile of proteins which were retained on Blue Sepharose (ROBS) which were not also found in the PAGE profile of the proteins which eluted directly from the Blue Sepharose column (PBS) (Figure 26).

To examine the mechanism by which uESP stimulated the outputs of PGs from Day-7 guinea-pig endometrium, the effect of uESP and other protein fractions purified from Day-15 guinea-pig endometrium were examined on the activity of PLA_2.

3:3:f The effects of proteins secreted by Day-15 guinea-pig endometrium on the activity of PLA_2

Introduction

As the activity of PLA_2 is the rate-limiting step in PG synthesis, any protein affecting the release of PGs from the guinea-pig endometrium may be acting by stimulating the activity of endometrial PLA_2. Therefore, in order to investigate the mechanism by which uESP stimulated PG output from Day-7 guinea-pig endometrium in culture, it was decided to examine the effect of uESP and the other protein fractions which had been purified by affinity, ion-exchange and gel filtration chromatography on the activity of PLA_2 in an *in vitro* assay.

Methods

Phosphatidylcholine (PC) with [^{14}C]-arachidonic acid (AA) in the 2-position was used as a radioactive substrate for PLA_2 from *Naja naja* venom. 0.5U/ml of PLA_2 was used as this concentration of PLA_2 gives a conversion of [^{14}C]-PC to [^{14}C]-AA of approximately 65% over a 10 min incubation period (Figure 5) allowing either stimulation or inhibition of PLA_2 to be determined.

1mg each of F1-F7 and uESP were dissolved in 1ml of PLA_2 assay buffer containing 0.5U/ml PLA_2 and 300μl of each protein solution were dispensed in triplicate into Eppendorf tubes. 3 controls containing 300μl of PLA_2 assay buffer (0.5U/ml) with no protein were also prepared. The reaction was carried out as described in Section 2:7:(i). The fatty acid released during the reaction was separated from unmetabolised phospholipid on silica gel columns

--using two different solvent systems as eluents. The arachidonic acid was eluted in hexane/1,4-dioxan/glacial acetic acid (90:10:1) while the phosphatidylcholine was eluted in the more polar solvent, chloroform/methanol/water (65:35:4). The amount of radioactivity in each fraction was determined by counting in a liquid scintillation counter for 4 min. The conversion of [^{14}C]-PC to [^{14}C]-AA during a 10 min incubation, calculated as a percentage of the total radioactivity recovered, was used as a measure of PLA_2 activity. The above experiment was repeated with F7:2 and F7:3 (1mg/ml).

Statistical tests

Differences between treated and control groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

The activity of cobra venom PLA_2 was significantly ($P < 0.05$) stimulated by F1, F2 and F7 (Table 9). In addition, both the low (3) and intermediate (2) molecular weight fractions of F7 (F7:2 and F7:3) significantly ($P < 0.05$) stimulated the activity of PLA_2 (Table 9). None of the other proteins tested had any effect on the activity of PLA_2 activity at the concentration used (1mg/ml) (Table 9).

Conclusions

The protein fractions F3-F6 and uESP had no effect on the activity of PLA_2 in an *in vitro* assay. However, those proteins which eluted from the ion-exchange column at either end of the NaCl gradient, F1,

Table 9. The effects of the Day-15 guinea-pig endometrial secretory protein fractions uESP, F1-F7, F7:2 and F7:3 on the mean ± s.e.m. ($\underline{n}$ = 3) % conversion of [^{14}C]-PC to [^{14}C]-AA by cobra venom PLA_2.

Protein	% conversion of [^{14}C]-PC to [^{14}C]-AA
Control	64.3 ± 2.66
uESP	56.7 ± 6.94
F1	82.0 ± 2.65+
F2	81.3 ± 1.45+
F3	54.3 ± 1.73
F4	54.5 ± 3.68
F5	51.3 ± 6.37
F6	57.0 ± 4.05
F7	84.0 ± 1.53+
F7:2	92.3 ± 0.67+
F7:3	90.7 ± 0.88+

+Significantly ($\underline{P} < 0.05$) higher than the control value.

F2 and F7, stimulated the activity of PLA_2 by 20-30%. The PLA_2 stimulatory activity was also found in the intermediate (2) and low (3) molecular weight fractions of F7. Only 0.5mg of the high (1) molecular weight fraction of F7 was originally isolated and after analysis of the molecular weights of the proteins present in this fraction by SDS PAGE, none was left to test on the activity of PLA_2. Neither F1 or F2 was further purified by gel filtration chromatography so it could not be determined which protein in these fractions was responsible for the stimulation of PLA_2 activity.

3:3:g The effects of intracellular proteins from Day-15 guinea-pig endometrium on the output of prostaglandins from Day-7 guinea-pig endometrium in culture

Introduction

It is possible that the protein which mediates the increase in $PGF_{2\alpha}$ synthesis in the guinea-pig endometrium at the end of the cycle is not secreted from the endometrium but is an intracellular protein. Indeed, if the protein is a G-protein linked to PLA_2 or a G-protein which controls the release of intracellular calcium from the endoplasmic reticulum, it may be unlikely that it would be secreted. Therefore, the effects of intracellular proteins isolated from Day-15 guinea-pig endometrium have been examined on the release of PGs from Day-7 guinea-pig endometrium in culture.

Methods

Intracellular protein was isolated from the Day-15 guinea-pig endometrium by the methods used by Gupta and Braun (1990) to isolate phospholipase stimulatory protein (PLSP) from embryonic mouse genital tracts. The uterus was removed from a Day-15 guinea-pig and "opened" via a longitudinal incision. The endometrium was dissected away from the myometrium and placed in 10ml of ice cold culture medium containing 1% Nonidet P-40. The tissue was shredded and homogenized while replacing on ice at intervals. The tissue sample was sonicated in an ice-water bath for 5 min and centrifuged at 250 x g for 10 min. The supernatant was removed, partially purified by dialysis and desalting steps, and lyophylized (see Section 2:10). This procedure was repeated with endometrium from another Day-15 guinea-pig ommitting Nonidet P-40 from the medium. The molecular

weights of the proteins present in the fractions isolated in the presence of Nonidet P-40 (+NON) and in the absence of Nonidet P-40 (-NON) were determined by SDS PAGE (see Section 2:8).

The uteri were removed from five Day-7 guinea-pigs and under aseptic conditions, each uterine horn was "opened" by a longitudinal incision. The endometrium was dissected away from the myometrium and cut into 1-2mm^3 pieces. 6 petri dishes each containing 5-10mg (1-2mg dry weight) of endometrium were prepared from each uterus. Pairs of dishes were treated with cellular protein isolated in the presence of Nonidet P-40 (+NON; 135μg/ml), cellular protein isolated in the absence of Nonidet P-40 (-NON; 135μg/ml) and no protein (control). The culture was carried out in 3.5 cm diameter petri dishes containing 2ml of culture medium. The petri dishes were incubated at 37°C for 12h, and the medium was sampled and replaced with fresh medium containing the same protein treatments at 6h. The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each petri dish into separate preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was reweighed and the amount of dried endometrium from each dish was calculated. The output of PGs was calculated per mg dry weight of endometrium.

Statistical tests

Changes in the output of PGs with time and differences between control and treated groups were analysed by Student's *t* test or, if the differences between the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal

variances.

Results

29.5mg of dried cellular protein was recovered from the endometrium (60.2mg dry weight) of one guinea-pig when the protein was isolated in the presence of Nonidet P-40 (+NON). 10.4mg of dried cellular protein was recovered from the endometrium (55.6mg dry weight) of one guinea-pig when the protein was isolated in the absence of Nonidet P-40 (-NON). The PAGE profiles obtained from cellular protein from Day-15 guinea-pig endometrium isolated in the presence (+NON) and absence (-NON) of Nonidet P-40 are shown in Figure 36. There is a wide molecular weight range of stained protein bands in both fractions with particularly heavy staining in the region of the molecular weight marker for bovine serum albumin (67kDa). The blurring of the bands in the low molecular weight region of the +NON fraction may be an effect of Nonidet P-40.

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium were significantly ($P < 0.01$) increased at 6h and 12h by the presence of cellular protein which had been isolated in the presence of Nonidet P-40 (+NON; 135μg/ml) (Figure 37). However, if no Nonidet P-40 was used in the isolation process, cellular endometrial protein (-NON; 135μg/ml) had no effect on the output of PGs from Day-7 guinea-pig endometrium in culture at 6h or 12h (Figure 37).

Conclusions

The stimulation of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 outputs from Day-7 guinea-pig endometrium in culture caused by cellular protein from Day-15 endometrium could be attributed entirely to the presence

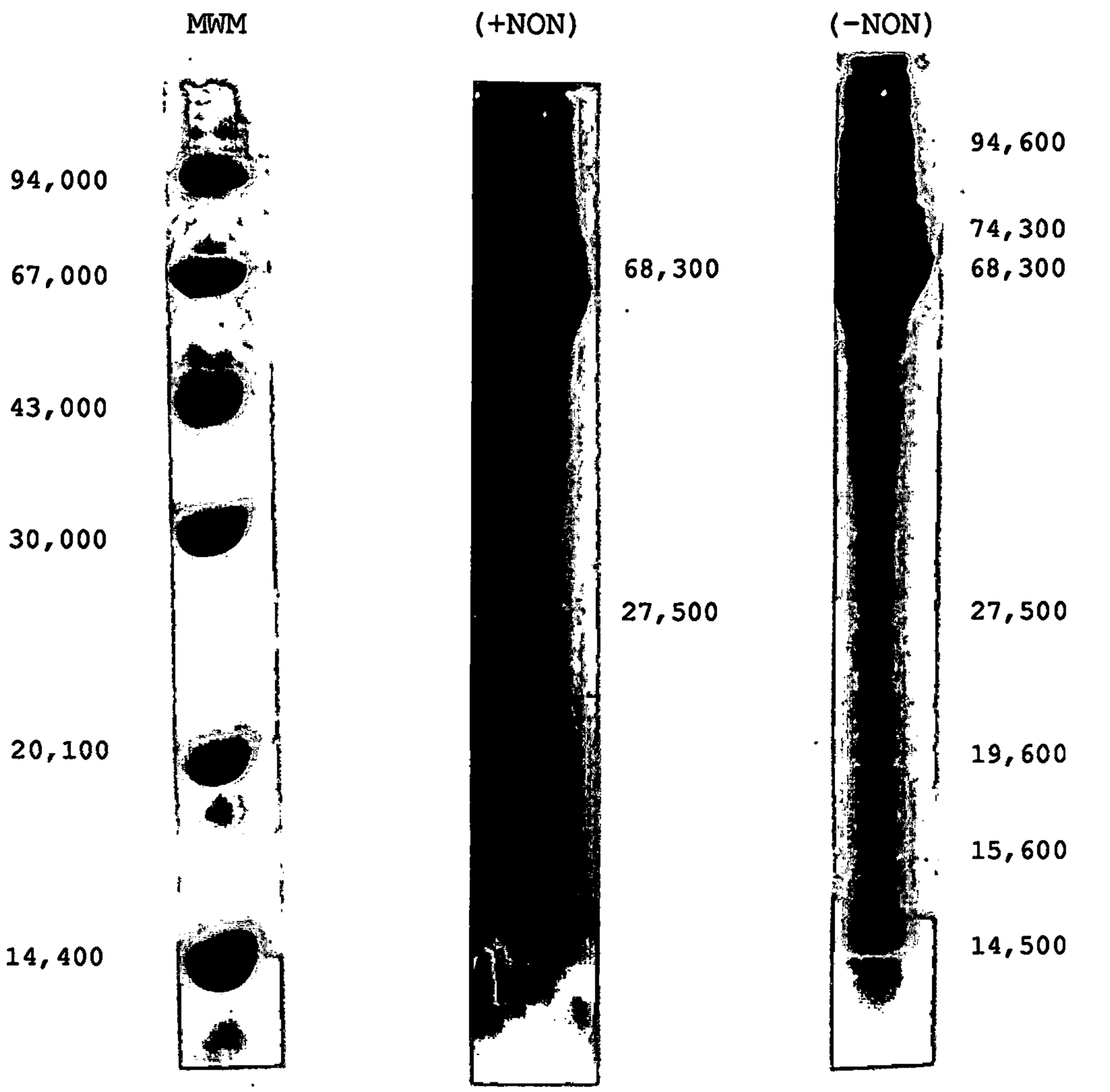


Fig. 36. The molecular weights (Da) of the major protein bands observed on the SDS PAGE proflies of cellular proteins from Day-15 guinea-pig endometrium isolated in the presence of Nonidet P-40 (+NON) and in the absence of Nonidet P-40 (-NON). The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 20µg of each protein fraction were run on the gel.

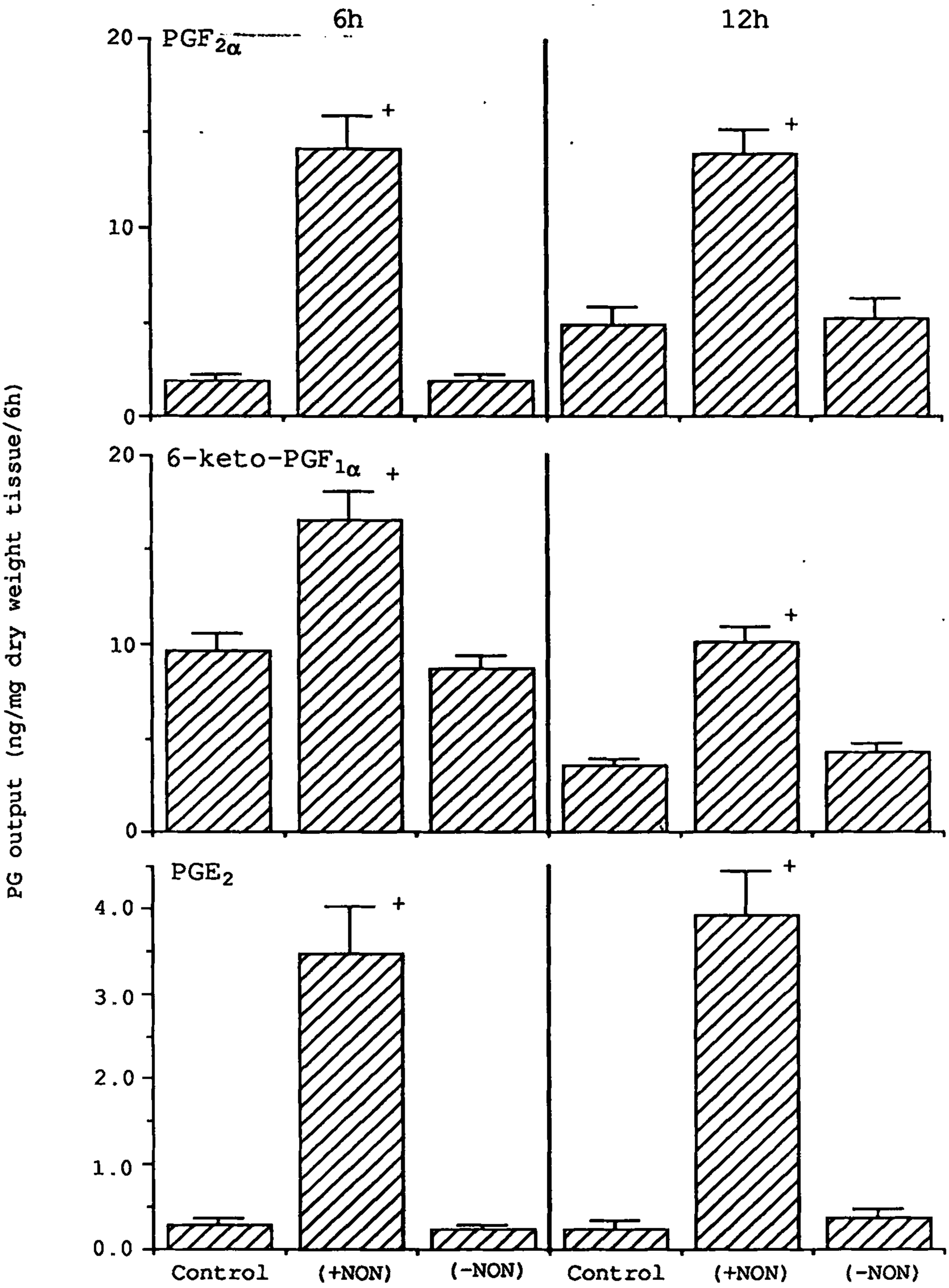


Fig. 37. The effects of cellular protein (135µg/ml) from Day-15 guinea-pig endometrium isolated, (i) in the presence of Nonidet P-40 (+NON), and (ii) in the absence of Nonidet P-40 (-NON) on mean (± s.e.m., $n = 10$) outputs of prostaglandins (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium cultured for 12h and sampled at 6h. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

of the nonionic surfactant Nonidet P-40 in the culture medium in which the protein was isolated, as the stimulation was not seen when cellular protein was isolated in the absence of Nonidet P-40. A possible mechanism by which the protein fraction isolated in the presence of Nonidet P-40 stimulates endometrial PG output was investigated by examining the effects of endometrial cellular protein isolated in the absence (-NON) and presence (+NON) of Nonidet P-40 on the activity of PLA_2.

3:3:h The effects of intracellular proteins from Day-15 guinea-pig endometrium on the activity of PLA_2

Introduction

The mechanism by which the cellular protein fraction isolated in the presence of Nonidet P-40 (+NON) may stimulate PG output from Day-7 guinea-pig endometrium was investigated by determining the effect of this protein fraction on PLA_2 activity. The cellular protein fraction isolated from Day-15 guinea-pig endometrium in the absence of Nonidet P-40 (-NON) was also examined for its effect on PLA_2 activity.

Methods

The PLA_2 assay was carried out using phosphatidylcholine (PC) with [^{14}C]-arachidonic acid (AA) in the 2-position as the labelled substrate. 1mg of the +NON and -NON fractions of cellular protein from Day-15 guinea-pig endometrium were dissolved in 1ml of PLA_2 assay buffer containing 0.5U/ml PLA_2 from Naja naja venom. This concentration of PLA_2 gives a conversion of [^{14}C]-PC to [^{14}C]-AA of approximately 65% during a 10 min incubation period (Figure 5) and allows for detection of either inhibition or stimulation of PLA_2 activity. 300μl of each protein solution were dispensed in triplicate into Eppendorf tubes. 3 controls were also prepared containing 300μl of PLA_2 assay buffer (0.5U/ml PLA_2) with no protein. The reaction was carried out as described in Section 2:7:(i). The unmetabolised substrate and the fatty acid were separated on silica gel columns using two different solvent systems. [^{14}C]-AA was eluted in hexane/1,4-dioxan/glacial acetic acid (90:10:1) and [^{14}C]-PC was eluted in the more polar solvent,

chloroform/methanol/water (65:35:4). The radioactivity in each solvent fraction was measured by counting in a liquid scintillation counter for 4 min. The conversion of [^{14}C]-PC to [^{14}C]-AA over a 10 min period, calculated as a percentage of the total radioactivity recovered, was used as a measure of PLA_2 activity.

Statistical tests

Differences between treated and control groups were analysed by Student's *t* test or, if the variances between the two groups were different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

The cellular protein fraction isolated in the presence of Nonidet P-40 (+NON; 1mg/ml) significantly ($P < 0.05$) inhibited the activity of cobra venom PLA_2 (Table 10). However the cellular protein fraction isolated in the absence of Nonidet P-40 (-NON; 1mg/ml) had no effect on the activity of cobra venom PLA_2 (Table 10).

Conclusion

The cellular protein fraction from Day-15 guinea-pig endometrium isolated in the presence of Nonidet P-40 resulted in the inhibition of the activity of PLA_2 from cobra venom in an *in vitro* assay compared to the control value and to the value for cellular protein isolated in the absence of Nonidet P-40

DISCUSSION

Protein isolated from culture medium from Day-15 guinea-pig endometrium was found to be contaminated with large amounts of

Table 10. The effects of Day-15 guinea-pig endometrial cellular protein, isolated in the presence (+NON) and absence (-NON) of Nonidet P-40, on the mean ± s.e.m. (n = 3) % conversion of [^{14}C]-PC to [^{14}C]-AA by cobra venom PLA_2.

Protein	% conversion of [^{14}C]-PC to [^{14}C]-AA
Control	66.07 ± 1.87
(+NON)	23.82 ± 5.34*
(-NON)	61.85 ± 0.86

*Significantly ($P < 0.05$) lower than the control value.

albumin (67.0kDa) from—serum. Large amounts of serum proteins are also found in uterine luminal fluid from the human (MacLaughlin, Santoro, Bauer, Lawrence and Richardson, 1986; Roberts, Parker and Henderson, 1976), pig (Murray et al., 1972), cow (Bartol et al., 1985), ewe (Roberts, Parker and Symonds, 1976b) and rabbit (Beier and Beier-Hellwig, 1973). That some of the proteins in the culture medium do originate from the endometrium has been shown by Abdi-Dezfuli and Poyser (1989) who found that [^{3}H]-leucine present in culture medium was incorporated into proteins secreted by the Day-15 guinea-pig endometrium, with higher incorporation of [^{3}H]-leucine occurring on Day 15 than on Day 7 of the cycle. This higher incorporation is consistent with the findings of Riley and Poyser (1989) who showed that the synthesis of secreted endometrial proteins was higher on Day 15 than on Day 7 of the cycle. Another serum contaminant of guinea-pig endometrial secretory proteins is transferrin (76.5kDa) which is also a predominant fraction, along with albumin, of rabbit uterine fluid (Beier and Beier-Hellwig, 1973).

Unpurified endometrial secretory protein (uESP; 700μg/ml) increased the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium by 9.4-, 1.5- and 4.3-fold respectively after 6h of culture. This compares with increases in the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 of 48.7-, 1.3- and 4.5-fold between Day-7 and Day-15 from endometrium in culture for 8h (see Section 3:1:a). Therefore, protein from the Day-15 endometrium stimulates the outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-7 endometrium to a similar extent as that seen in the Day-15 endometrium. However the stimulation of $PGF_{2\alpha}$ synthesis was not as great as that seen in the Day-15 endometrium. This could have been

due to the depletion of necessary cofactors, such as calcium, throughout the culture period which might be necessary to maintain the stimulation.

None of the proteins purified from uESP by affinity, ion-exchange or gel filtration chromatography was capable of stimulating the output of PGs from Day-7 guinea-pig endometrium in culture. However F1, F2, F7 and the intermediate (F7:2) and low (F7:3) molecular weight fractions of F7 did stimulate the activity of PLA_2 from cobra venom in an <u>in vitro</u> assay. F1, F2 and F7 all contained heavily stained bands < 20.0kDa when analysed by SDS PAGE which were not prominent bands in the PAGE profiles of F3, F4, F5 and F6 (Figure 28). As there were insufficient amounts of F1, F2, F7 and F7:3 to test on the output of PGs from the Day-7 guinea-pig endometrium it is not possible to tell whether the low molecular weight proteins present in these fractions which stimulate PLA_2 are responsible for the increase in $PGF_{2\alpha}$ output from the guinea-pig endometrium which occurs at the end of the cycle. However, the intermediate fraction (F7:2) of F7 which did stimulate PLA_2 activity <u>in vitro</u> failed to increase the outputs of PGs from Day-7 guinea-pig endometrium at a concentration of 20μg/ml. The fact that 1mg/ml of uESP had no effect on the activity of PLA_2 whereas 700μg/ml uESP greatly stimulated the output of PGs from the Day-7 guinea-pig endometrium in culture, suggests that either uESP stimulates PG output by a mechanism other than activation of PLA_2, or that the factors which regulate the activity of guinea-pig endometrial PLA_2 in culture are different from the factors which control the activity of PLA_2 from cobra venom in an <u>in vitro</u> assay. Therefore, the stimulation of cobra venom PLA_2 in an <u>in vitro</u> assay by F1, F2, F7, F7:2 and F7:3 does not necessarily

indicate that these proteins would have a similar effect on the activity of guinea-pig endometrial PLA_2 activity.

Another possibility is that the stimulation of PG output from the Day-7 endometrium caused by uESP was a nonspecific effect of the large amount of protein used (700μg/ml). PLA_2 is inhibited by both lysophosphatidylcholine and fatty acids and this product-inhibition of PLA_2 is prevented by high concentrations of bovine serum albumin (Smith, Gul and Thompson, 1972; Kupferberg, Yokoyama and Kezdy, 1981; Pluckthun and Dennis, 1985). A lipid water interface is created between water-soluble PLA_2 and phospholipid when the phospholipid concentration reaches the critical concentration necessary to form micelles. At this time there is an abrupt increase in the activity of PLA_2. Fatty acids provide charge at the lipid/water interface and inhibit the activity of PLA_2. Therefore the high concentration of uESP used may have bound fatty acids released by PLA_2 and prevented them from disturbing the lipid/water interface. The large amount of albumin present in uESP may have been responsible for this effect or it may have been a nonspecific effect of the proteins present in uESP since gamma-globulin exhibited a similar relief of product-inhibition of PLA_2 as bovine serum albumin (Conricode and Ochs, 1989).

The cellular protein fractions from Day-15 endometrium contained more proteins in the intermediate and low molecular weight regions than fractions of proteins secreted by Day-15 guinea-pig endometrium. However cellular proteins isolated from Day-15 endometrium were incapable of stimulating PLA_2 output from Day-7 guinea-pig endometrium in culture.

The protein fraction isolated in the presence of the nonionic detergent Nonidet P-40 was found to stimulate the output of PGs from

Day-7 guinea-pig endometrium in culture. This may be attributable directly to the presence of Nonidet P-40. Ionic detergents can affect PLA_2 activity by providing charge at the lipid/water interface between PLA_2 and phospholipids. Indeed, the stimulatory action of the bee venom protein, melittin, on PLA_2 activity is thought to be due to the similarity of its structure to a cationic detergent (Yunes, Goldhammer, Garner and Cordes, 1977). However Nonidet P-40 is a nonionic detergent and should not therefore affect the charge at the lipid/water interface of PLA_2 and phospholipids. The nonionic detergent Triton X-100, which forms micelles in aqueous solution, is used to convert phospholipid bilayers, which are poor substrates for PLA_2, into mixed Triton X-100-phospholipid micelles (Dennis, 1973a) which are much more susceptible to enzymatic attack by PLA_2. Nonidet P-40 may have a similar effect on lipid bilayers in the guinea-pig endometrium thus increasing the activity of endometrial PLA_2 and stimulating PG synthesis. However the protein fraction isolated in the presence of Nonidet P-40 was found to inhibit the activity of cobra venom PLA_2 <u>in vitro</u>. These results suggest that either the increase in PG output from Day-7 guinea-pig endometrium in culture caused by the Nonidet P-40-containing protein fraction is not due to an effect on endometrial PLA_2 activity or that cobra venom PLA_2 and guinea-pig endometrial PLA_2 are affected differently by the Nonidet P-40-containing fraction. High concentrations of the nonionic detergent, Triton X-100, have been shown to inhibit PLA_2 activity in Triton X-100-phospholipid mixed micelles, possibly by a surface dilution effect (Dennis, 1973b). The concentrations of Nonidet P-40 used in the PLA_2 assay may have inhibited PLA_2 activity in a similar manner.

3:4 INVESTIGATIONS INTO THE INVOLVEMENT OF INTERFERON IN THE PREVENTION OF LUTEAL REGRESSION IN THE PREGNANT GUINEA-PIG

3:4:a The effect of α-interferon on the output of prostaglandins from Day-15 guinea-pig endometrium in culture

Introduction

The continued secretion of progesterone is a necessary prerequisite for the maintenance of pregnancy. As the initial source of progesterone is the corpus luteum and as in eutherian mammals pregnancy usually lasts longer than the period of luteal activity in an oestrous cycle, essential requirements for the establishment and maintenance of pregnancy are that the ovarian cycle be changed and the functional activity of the corpus luteum be prolonged. The regression of the corpus luteum during the oestrous cycle is associated with the release of $PGF_{2\alpha}$ from the uterine endometrium in many species of non-primate mammals (see Horton and Poyser, 1976). The presence of a conceptus within the uterus attenuates the secretion of $PGF_{2\alpha}$ from the endometrium (Poyser, 1981). Therefore, at some time prior to the normal period of luteal regression, the blastocyst must signal its presence by releasing either a luteotrophic substance to act on the ovary and antagonise the action of $PGF_{2\alpha}$ or an antiluteolytic substance which acts on the uterus to prevent $PGF_{2\alpha}$ output.

In some species of domestic ruminant (e.g. the cow, sheep and pig), the signal for pregnancy recognition is transmitted to the mother even before the embryonic tissue becomes intimately attached to the uterine epithelia and is, therefore, clearly distinct from implantation. The blastocysts of these species, while free in the uterine lumen, must produce some substance that is capable of

diffusion into the uterine fluids and across the lumen to exert a local effect on the uterine endometrium. The major secretory product of Day-13 sheep conceptuses is a low molecular weight, acidic protein (Godkin et al., 1982a). The purified protein, named ovine trophoblast protein-1 (oTP-1), extends luteal function in non-pregnant sheep, binds to receptors in the ovine endometrium (Godkin et al., 1984b), inhibits $PGF_{2\alpha}$ release in response to oxytocin and oestradiol in cyclic ewes (Vallet et al., 1987a), and inhibits $PGF_{2\alpha}$ and PGE_2 release from ovine endometrial cells in culture (Salamonsen et al., 1988).

Day 16-18 bovine conceptus secretory proteins (bCSP) also extend interoestrous intervals (Knickerbocker et al., 1986a) and attenuate uterine PGF production in response to oestradiol (Knickerbocker, Thatcher, Barron and Roberts, 1986b) when infused into cyclic cows. Anti-serum to oTP-1 cross reacts immunologically with some of the low molecular weight, acidic proteins of bCSP (Helmer et al., 1987). This immunoprecipitated complex comprises of 7 isomers of N-linked glycoproteins (Anthony, Helmer, Sharif, Roberts, Hansen, Thatcher and Bazer, 1988) known as bovine trophoblast protein-1 (bTP-1).

Anti-serum to oTP-1 also immunoprecipitates a low molecular weight acidic protein secreted by Day 16-21 goat conceptuses (Gnatek et al., 1989). Thus oTP-1, bTP-1 and cTP-1 (caprine trophoblast protein-1) are immunologically alike.

Maternal recognition of pregnancy in pigs is thought to be brought about by the secretion of oestrogens by the conceptus which redirects the secretion of PGF towards the uterine lumen (Bazer and Thatcher, 1977). Although pig conceptuses secrete two major classes of protein between Days 10.5 and 18 of gestation (Godkin et al., 1982a), infusion of porcine conceptus secretory proteins (pCSP)

into the uterine lumen of cyclic pigs has no effect on interoestrous intervals or progesterone levels (Harney and Bazer, 1989). pCSP do not therefore appear to play a role in the maternal recognition of pregnancy in the pig.

oTP-1 exhibits high amino acid sequence homology with interferons of the α-2 class (Stewart et al., 1987; Imakawa et al., 1987; Charpigny et al., 1988). α-Interferon binds to oTP-1 receptors in ovine endometrium (Stewart et al., 1987; Flint et al., 1988), and extends luteal phase lengths and lowers PGFM concentrations in cyclic ewes (Lamming et al., 1988). α-Interferon also inhibits $PGF_{2\alpha}$ and PGE_2 synthesis in cultured ovine endometrial cells (Salamonsen et al., 1988) with 100 times the potency of oTP-1 (Salamonsen et al., 1989). Recombinant bovine α-interferon extends the interoestrous interval when infused into cyclic cows (Plante et al., 1988) and bTP-1 shares high sequence homology with α-interferons (Imakawa et al., 1989).

Day-11 porcine conceptuses produce a cluster of low molecular weight acidic proteins which cross-react with antiserum to human α-interferon but not with antiserum to oTP-1 (Cross and Roberts, 1989). Therefore, although porcine conceptuses secrete interferon-like proteins, they are not immunologically similar to oTP-1.

In the guinea-pig, ovarian progesterone is necessary for the maintenance of pregnancy for the first 4-5 weeks (Heap and Deanesly, 1966; Csapo et al., 1981). The increase in concentration of $PGF_{2\alpha}$ in uterine venous plasma after Day 11 in cyclic guinea-pigs does not occur in pregnant guinea-pigs (Blatchley et al., 1975a, 1975b; Antonini et al., 1976). However, this is not due to redirection of $PGF_{2\alpha}$ secretion towards the uterine lumen as proposed for the pig

(Bazer and Thatcher, 1977) as the output of $PGF_{2\alpha}$ from the guinea-pig uterus superfused *in vitro* is much lower on Day 15 of pregnancy than on Day 15 of the cycle (Poyser, 1984a).

The grafting of Day 11 or 12 but not Day 6, 9 or 10 guinea-pig conceptuses onto the spleen of non-pregnant guinea-pigs extends the oestrous cycle of the recipients (Bland and Donovan, 1969b), suggesting that the guinea-pig conceptus produces a systemically acting substance from Day 11 which suppresses $PGF_{2\alpha}$ release from the uterine endometrium and prevents corpus luteum regression. The identity of the antiluteolytic factor from the guinea-pig conceptus is unknown. To determine whether a similar mechanism for maternal recognition of pregnancy to that which occurs in the sheep, cow and goat, due to the release of interferon-like oTP-1, bTP-1 and cTP-1, exists in the guinea-pig, the effects of human α-interferon on PG synthesis by guinea-pig endometrium have been investigated. The effects of two known inhibitors of endometrial $PGF_{2\alpha}$ output (namely indomethacin and long-term treatment with sodium fluoride) which act in different ways were also studied for control purposes.

Methods

The uteri were removed from five Day-15 guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 12 petri dishes containing 15-30mg wet weight (3-6mg dry weight) of endometrium were prepared from each uterus. Pairs of dishes were treated with 5U/ml human α-interferon, 50U/ml human α-interferon, 2mM sodium fluoride, 10mM sodium fluoride, 2μg/ml indomethacin and no treatment (control). The petri dishes were incubated at 37°C for 24h, and the

culture medium was removed and replaced with fresh medium containing the same treatment every 6h. The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture the pieces of endometrium were removed from each petri dish into preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was reweighed and the amount of dried endometrium from each petri dish was calculated. This experiment was repeated using α-interferon at concentrations of 500 and 2500 U/ml instead of 5 and 50 U/ml. The outputs of PGs were calculated per mg dry weight of endometrium.

Statistical tests

Changes in the output of PGs with time were analysed by Duncan's multiple range test or, if the variances of the groups were significantly different by the variance ratio F test, by a modified t test for unequal variances. Differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

The control outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium significantly ($P < 0.05$) declined during 24h of culture (Figure 38). The control inhibitor indomethacin (2μg/ml) significantly ($P < 0.05$) inhibited the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium after 6, 12, 18 and 24h of culture (Figure 38).

Human α-interferon (5 and 50U/ml) had no effect on the outputs of

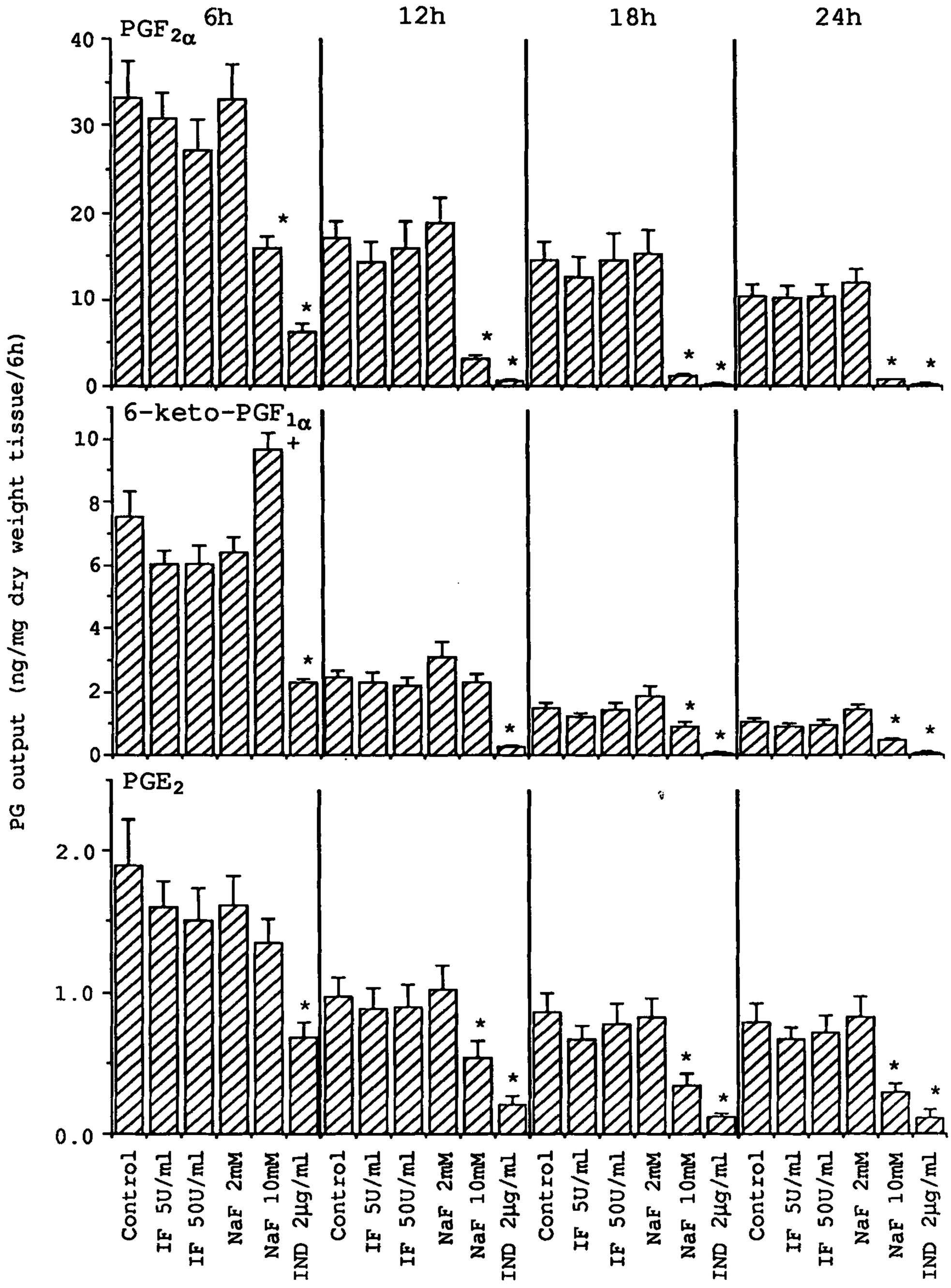


Fig. 38. Effects of human α-interferon (IF; 5 and 50U/ml), sodium fluoride (NaF; 2 and 10mM) and indomethacin (IND; 2μg/ml) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium cultured for 24h and sampled every 6h. *Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium in-culture for 24h at any time period studied (Figure 38).

Sodium fluoride (10mM but not 2mM) significantly ($\underline{P} < 0.05$) inhibited $PGF_{2\alpha}$ output after 6h, $PGF_{2\alpha}$ and PGE_2 outputs after 12h, and $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 outputs after 18 and 24h from Day-15 endometrium in culture (Figure 38). Sodium fluoride (10mM but not 2mM) significantly ($\underline{P} < 0.05$) stimulated 6-keto-$PGF_{1\alpha}$ output from Day-15 endometrium after 6h of culture (Figure 38).

When this experiment was repeated using higher concentrations of human α-interferon (500 and 2500U/ml instead of 5 and 50U/ml), there was still no effect of human α-interferon on the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium in culture for 24h at any time period studied (Figure 39). The results for indomethacin (2μg/ml) and sodium fluoride (2mM and 10mM) (Figure 39) replicated exactly those found above (Figure 38).

Conclusions

Human α-interferon at concentrations of 5, 50, 500 and 2,500U/ml had no effect on the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium in culture over 24h. Consequently, it appears that the antiluteolytic factor secreted by the guinea-pig conceptus is probably not an interferon and is therefore different from oTP-1.

Indomethacin (2μg/ml) significantly inhibited the output of PGs by Day-15 guinea-pig endometrium in culture at all time periods showing that PG synthesis by guinea-pig endometrium occurred during tissue culture and indicating that the culture system is sensitive to measuring a decrease in endometrial PG synthesis. In addition, 10mM

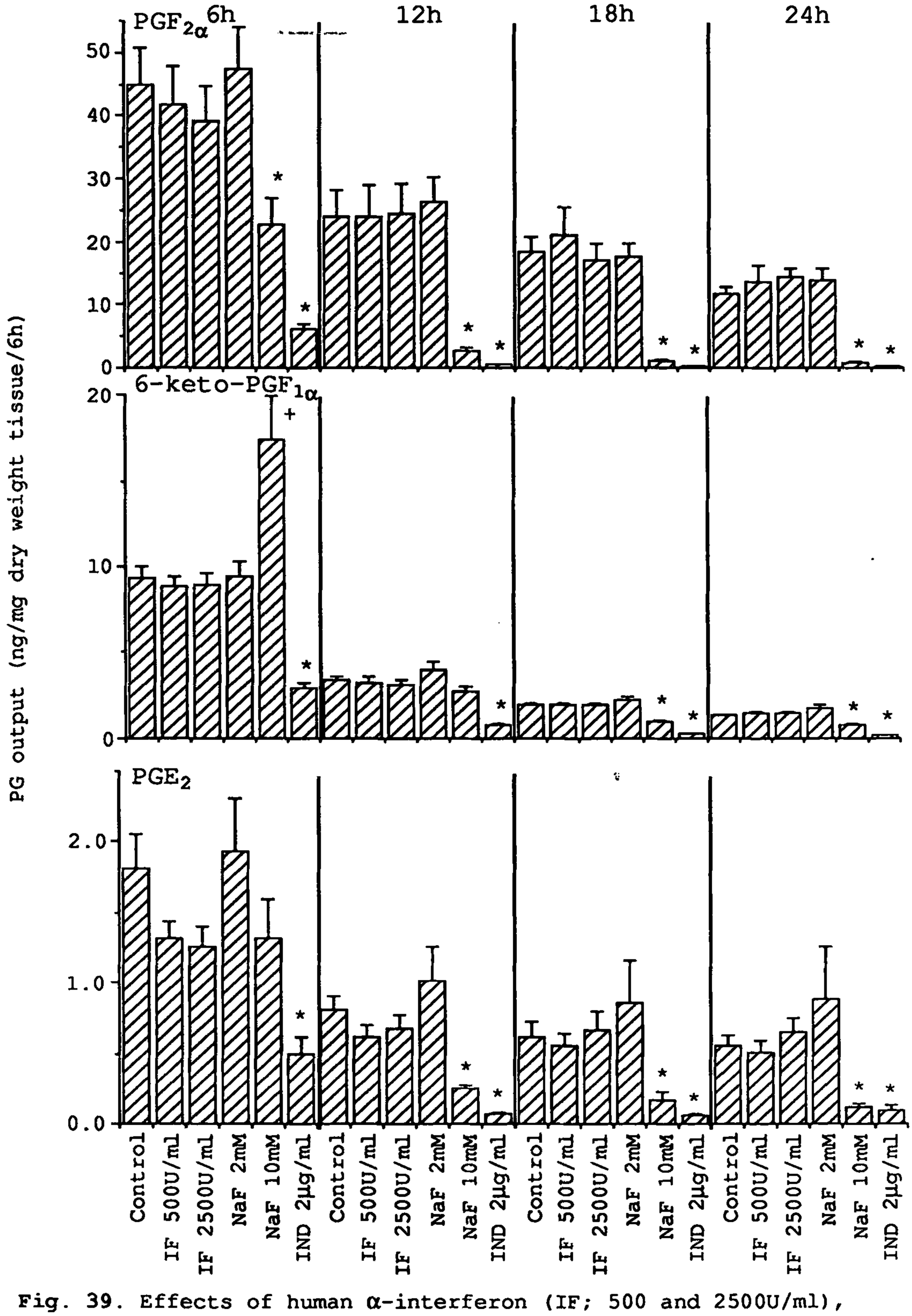


Fig. 39. Effects of human α-interferon (IF; 500 and 2500U/ml), sodium fluoride (NaF; 2 and 10mM) and indomethacin (IND; 2μg/ml) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium cultured for 24h and sampled every 6h.*Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time. +Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

sodium fluoride inhibited $PGF_{2\alpha}$ output from Day-15 cultured guinea-pig endometrium after 6h, $PGF_{2\alpha}$ and PGE_2 outputs after 12h and $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ outputs after 18h and 24h confirming the results found in Section 3:1:a. The stimulatory effect of 10mM sodium fluoride on the production of 6-keto-$PGF_{1\alpha}$ from Day-15 guinea-pig endometrium in culture after 6h reconfirmed that 6-keto-$PGF_{1\alpha}$ is either more sensitive to the stimulatory effects of sodium fluoride or less sensitive to the long-term inhibitory effects of sodium fluoride and may have its release controlled by a different mechanism than that for $PGF_{2\alpha}$ and PGE_2.

3:4:b Assay of guinea-pig conceptus protein for the presence of antiviral activity

Introduction

The antiluteolytic proteins secreted by the sheep, cow and goat conceptus, known as oTP-1, bTP-1 and cTP-1 respectively, are functionally and immunologically similar to interferons of the α family (Stewart et al., 1987; Charpigny et al., 1988; Imakawa et al., 1988; Salamonsen et al., 1988; Gnatek et al., 1989). oTP-1 exhibits antiviral activity comparable to that of α-interferons (about 10^7-10^8 relative U/mg of protein) (Pontzer, Torres, Vallet, Bazer and Johnson, 1988). As expected, total bovine conceptus secretions (Betteridge, Derbyshire, Rorie, Scodras and Johnson, 1988) and purified bTP-1 (Godkin et al., 1988b) also have antiviral activity. Secretory proteins from the Day-15 guinea-pig conceptus were therefore assayed for antiviral activity indicative of interferon to determine if interferon-like proteins are involved in the antiluteolytic mechanism in the pregnant guinea-pig.

Methods

A virgin female guinea-pig, which had undergone at least 3 normal oestrous cycles was mated by placing with a male guinea-pig several days prior to oestrus. The guinea-pig was examined daily for signs of mating and for the presence of sperm in the vaginal smear, with Day 1 of pregnancy being the first day of finding any of these signs.

On Day-15 of pregnancy, the guinea-pig was killed and the uterus removed. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the conceptuses were gently removed.

Each conceptus was cut into 1-2mm^3 pieces and 8 petri dishes were prepared using the conceptus tissue (a total of 74.9mg dry weight). The petri dishes were incubated at 37°C for 24h. The culture medium was removed from each dish and pooled before undergoing dialysis and desalting (see Section 2:10). The tissue was removed from each dish and amalgamated in a preweighed container. The tissue was dried by placing in an oven at 37°C for 24h. The container was reweighed and the amount of dried conceptus tissue was calculated.

The dialysed and desalted medium (3.5ml) was frozen and sent on dry ice to the Institute of Zoology, Regent's Park, London where it was assayed for antiviral activity by Professor A.P.F. Flint.

Results

The medium obtained by incubating the Day-15 guinea-pig conceptus for 24h was found to contain no detectable antiviral activity. The detection limit of the assay was 16U/ml.

Conclusions

There was no detectable antiviral activity indicative of interferon or interferon-related substances being secreted by the Day-15 guinea-pig conceptus. Consequently, it is unlikely that the secretion of an interferon-like protein by the conceptus is involved in mediating the antiluteolytic mechanism in the pregnant guinea-pig.

DISCUSSION

Human α-interferon in concentrations up to 500 times greater than those which are effective in inhibiting $PGF_{2\alpha}$ and PGE_2 output from ovine endometrial cells (Salamonsen et al., 1988, 1989), had no

effect on PG output from Day-15 guinea-pig endometrium in culture. Present and previous studies have shown that indomethacin, protein synthesis inhibitors, calmodulin antagonists, TMB-8 (an intracellular calcium antagonist), EGTA and lack of extracellular calcium inhibit PG output from Day-15 guinea-pig endometrium in culture (Riley and Poyser, 1987a, 1987b, 1989), indicating that the culture technique used is particularly sensitive for detecting the various ways in which endometrial PG synthesis can be inhibited. Therefore, it appears that interferons or interferon-like substances are not involved in preventing the increase in endometrial $PGF_{2\alpha}$ synthesis, which normally occurs at the end of the cycle, in the pregnant guinea-pig. Supporting this conclusion was the finding that there were no detectable levels (>16U/ml) of antiviral activity (indicative of interferon) secreted into culture medium by Day-15 guinea-pig conceptuses. This is in contrast to the levels of antiviral activity of approximately 10^7U/ml in uterine flushings from the sheep on Day-16 of pregnancy (A.P.F. Flint, personal communication). Consequently, secretion of an interferon-like protein by the conceptus does not appear to be responsible for the prevention of luteolysis in the pregnant guinea-pig. Neither the horse conceptus (Sharp et al., 1989) nor the mouse trophoblast (Baker and Neider, 1990) secrete antiviral activity indicative of interferon, and it may be that this mechanism of delaying luteal regression during pregnancy by the secretion from the embryo of α-interferon related proteins is limited to domestic ruminants. Antiviral activity is present in porcine conceptus culture medium and flushings from Day 11-17 of pregnancy but the proteins found are not similar to oTP-1 (Cross and Roberts, 1989). Human foetal blood, organs, placenta, membranes, amniotic fluid and decidua all contain

significant amounts of interferon-α (Chard, Craig, Menabawey and Lee, 1986), and there is a progressive increase in the level of interferon in the mouse placenta during gestation (Fowler, Reed and Giron, 1980). The placenta is an organ with protective endocrine and immunological functions and occupies the intermediate zone between the maternal and fetal organisms. Placental interferon in the human and mouse may therefore play a role in the immunoregulation of foetal acceptance.

The mechanism by which blastocyst interferon acts to reduce uterine $PGF_{2\alpha}$ secretion in the pregnant sheep and cow is thought either to be due to a direct effect on oxytocin receptor concentrations or by inducing the synthesis of an intracellular endometrial inhibitor of PG synthesis. In addition, Chandrabose and Cuatrecasas (1981) have shown that treatment of mouse S-180 sarcoma cells with mouse fibroblast (β) interferon causes the reduction of the unsaturated fatty acid content of all major phospholipids, resulting in a membrane where the ratio of saturated to unsaturated fatty acids is increased. Arachidonate is consistently reduced in all phospholipid classes after interferon treatment suggesting that blastocyst interferons may reduce PG synthesis by depleting precursor arachidonic acid. However this effect was not seen when human lymphoblastoid (α) interferon was used and may be a particular effect of β interferon alone.

There is a possibility that the human α-interferon used in this experiment may not exhibit close enough structural homology with any interferon-like antiluteolytic factor from the guinea-pig conceptus, in order to inhibit PG output from the guinea-pig endometrium. Indeed, recombinant bovine interferon-α1 has been shown to exert an antiluteolytic effect via a mode of action different from that of

bTP-1 (Thatcher, Hansen, Gross, Helmer, Plante and Bazer, 1989). However, both oTP-1 and bTP-1 exhibit 45-55% sequence homology with interferons of the α family from such diverse species as the human, mouse, rat and pig suggesting that any guinea-pig interferon would likely to be significantly similar to the interferons of other species as well.

The human and guinea-pig blastocysts implant within the first week after fertilisation, whereas in the domestic ruminant implantation does not occur for several weeks. Accordingly, the mechanism of maternal recognition of pregnancy may differ between those species which implant early and those species in which the embryos have to signal their presence prior to implantation. Indeed, the synthesis of $PGF_{2\alpha}$ by human endometrial cell cultures is unaffected by human interferon α-2 at concentrations of 5-500U/ml (Mitchell and Smith, 1989). This suggests that, like the guinea-pig, the maternal recognition of pregnancy in the human is not mediated by an interferon-like factor by the conceptus.

3:5 INVESTIGATIONS INTO THE EFFECTS OF SECRETIONS FROM THE DAY-15 GUINEA-PIG CONCEPTUS AND DAY-15 PREGNANT GUINEA-PIG ENDOMETRIUM ON THE OUTPUT OF PROSTAGLANDINS FROM GUINEA-PIG ENDOMETRIUM IN CULTURE

3:5:a The effect of co-culturing with Day-15 guinea-pig conceptus tissue on the output of prostaglandins from Day-15 non-pregnant guinea-pig endometrium in culture

Introduction

The previous experiments (Section 3:4) suggested that the antiluteolytic factor produced by the guinea-pig conceptus (Bland and Donovan, 1969b; Poyser, 1984a) is not an interferon and therefore probably not related to oTP-1 or bTP-1. PGF production by endometrial explants of mares *in vitro* has been shown to be significantly reduced during incubation with equine conceptus membranes (Berglund, Sharp, Vernon and Thatcher, 1982; Sharp, Zavy, Vernon, Bazer, Thatcher and Berglund, 1984; Sharp and McDowell, 1985). Therefore, the following experiment has examined the effect of Day-15 guinea-pig conceptus tissue on the production of PGs from Day-15 non-pregnant endometrium in culture. Day 15 is normally a day of high $PGF_{2\alpha}$ production by the guinea-pig endometrium in culture (Riley and Poyser, 1986), and it was expected that any antiluteolytic factor released into the medium by the conceptus tissue might be able to inhibit this production.

Methods

Five virgin female guinea-pigs which had undergone at least 3 normal oestrous cycles, were mated by placing with male guinea-pigs several days prior to oestrus. Guinea-pigs were examined daily for

signs of mating and for the presence of sperm in the vaginal smear, with Day 1 of pregnancy being the first day of finding any of these signs.

The uteri were removed from five Day-15 non-pregnant guinea-pigs and five Day-15 pregnant guinea-pigs. Under aseptic conditions, each uterus was "opened" via a longitudinal incision. The conceptuses were gently removed from the pregnant uteri and cut into 1-2mm^3 pieces. The endometrium was dissected away from the myometrium of the non-pregnant uteri and cut into 1-2mm^3 pieces. 18 petri dishes were prepared using the tissue from one pregnant and one non-pregnant animal as follows: 6 dishes containing 15-30mg wet weight (3-6mg dry weight) of endometrial tissue alone (control endometrium), 6 dishes containing 15-30mg wet weight (3-6mg dry weight) of conceptus tissue alone (control conceptus), and 6 dishes containing 15-30mg wet weight (3-6mg dry weight) of endometrial tissue and 15-30mg wet weight (3-6mg dry weight) of conceptus tissue together (co-culture). In addition, a further 3 dishes were prepared containing 50-90mg wet weight (10-18mg dry weight) of conceptus tissue. 9 of the petri dishes (3 from each treatment group) were incubated at 37°C for 12h with sampling and replacement with fresh medium at 6h. The culture medium from the petri dishes containing the co-cultured endometrial and conceptus tissue was replaced with medium from the 3 extra dishes of conceptus tissue prepared and incubated for 6h. The other 9 petri dishes were incubated at 37°C for 12h continuously with no sampling.

The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of tissue from each dish were removed into preweighed containers and

dried by placing in an oven at 37°C for 24h. Each container was reweighed and the amount of dried tissue from each petri dish was calculated. The endometrial and conceptus tissue from each of the co-culture dishes was dried and weighed separately. The output of PGs was calculated per mg dry weight of endometrium or conceptus for dishes containing endometrium or conceptus tissue alone respectively, and per mg dry weight of endometrium for the co-culture dishes. The amounts of PGs found in the co-culture dishes were also corrected for the production of PGs by the conceptus and, for the dishes which underwent replacement of medium at 6h, for the presence of PGs in the replacement medium, by using the data for PG output from conceptus tissue alone.

Statistical tests

Changes in PG output with time and differences between treated and control groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

The control outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium significantly ($P < 0.05$) declined during 12h of culture (Figure 40). The control output of $PGF_{2\alpha}$ from Day-15 endometrium was unchanged during 12h of culture (Figure 40). The control outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from Day-15 guinea-pig conceptus significantly ($P < 0.05$) declined during 12h of culture (Figure 40). The control output of PGE_2 from Day-15 conceptus was unchanged during 12h of culture (Figure 40).

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 were

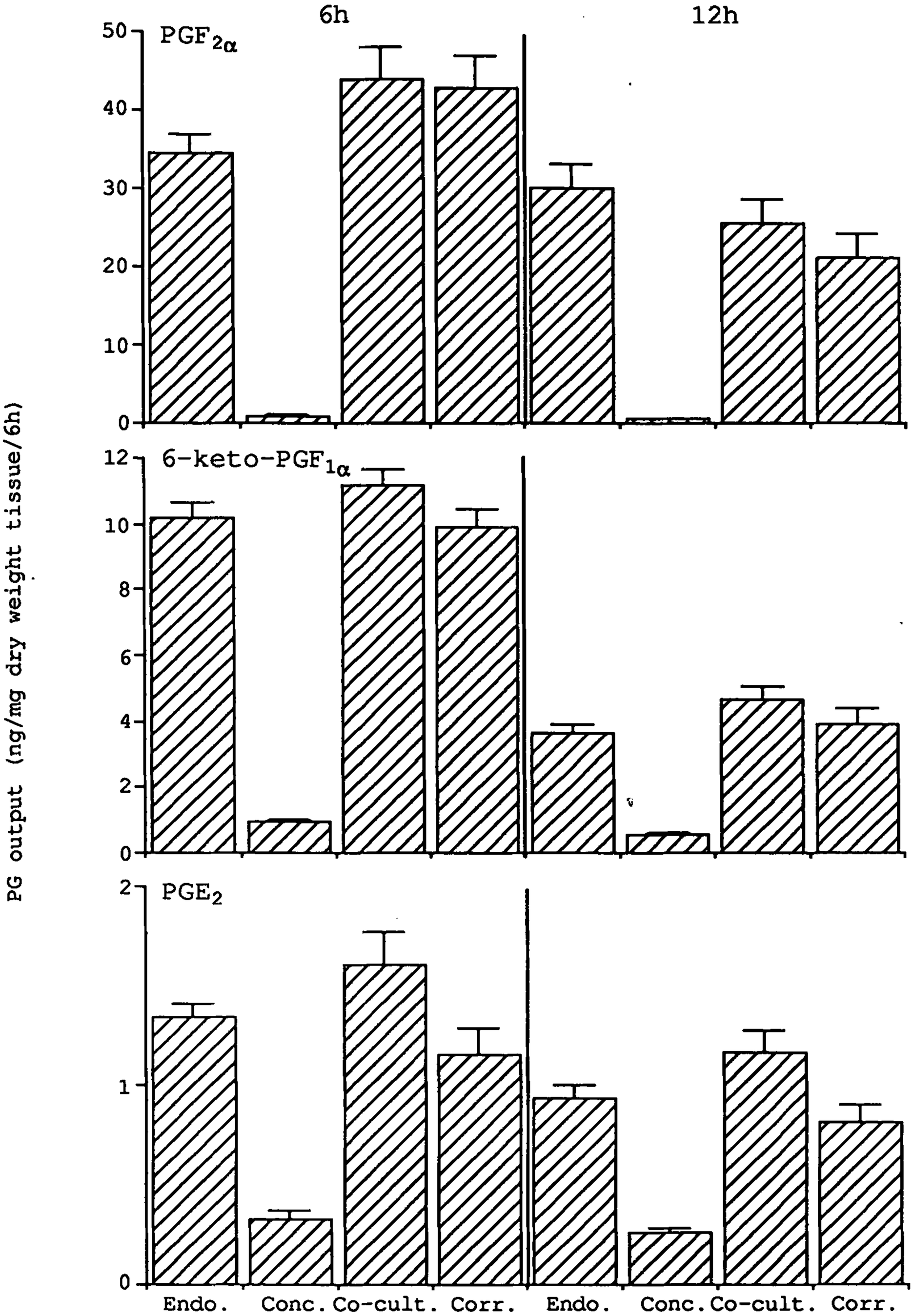


Fig. 40. The outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium (Endo.), Day-15 conceptus (Conc.), and from Day-15 endometrium cultured (i) together with Day-15 conceptus (Co-cult.) and (ii) after correcting (Corr.) for the amounts of PGs produced by the Day-15 conceptus, cultured for 12h with sampling at 6h. Results are mean ± s.e.m. (n = 15).

significantly ($P < 0.01$) higher from Day-15 endometrium than from Day-15 conceptus tissue at all time periods studied (Figures 40 and 41).

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium co-cultured with Day-15 conceptus (co-culture) tended to be higher during the first 6h of culture compared to the control outputs from Day-15 endometrium (Figure 40). However, this stimulation was not significant and could be accounted for by conceptus PG production (Figure 40).

The output of $PGF_{2\alpha}$ from Day-15 guinea-pig endometrium co-cultured with Day-15 conceptuses between 6h and 12h of culture was not significantly different from that of Day-15 control endometrium between 6h and 12h even after correction for conceptus PG production (Figure 40). The outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium co-cultured with Day-15 conceptuses tended to be higher between 6h and 12h of culture compared to the control outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium (Figure 40). However, this stimulation was not significant and could be accounted for by conceptus PG production (Figure 40).

The outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium co-cultured with Day-15 conceptus for 12h, with no change of medium, were not significantly different from the control Day-15 endometrium either before or after correction for PG production by the conceptus (Figure 41). The output of PGE_2 from Day-15 endometrium co-cultured with Day-15 conceptus for 12h, with no change of medium, was significantly ($P < 0.05$) decreased compared to the output from control Day-15 endometrium both before and after correction for conceptus PG production (Figure 41).

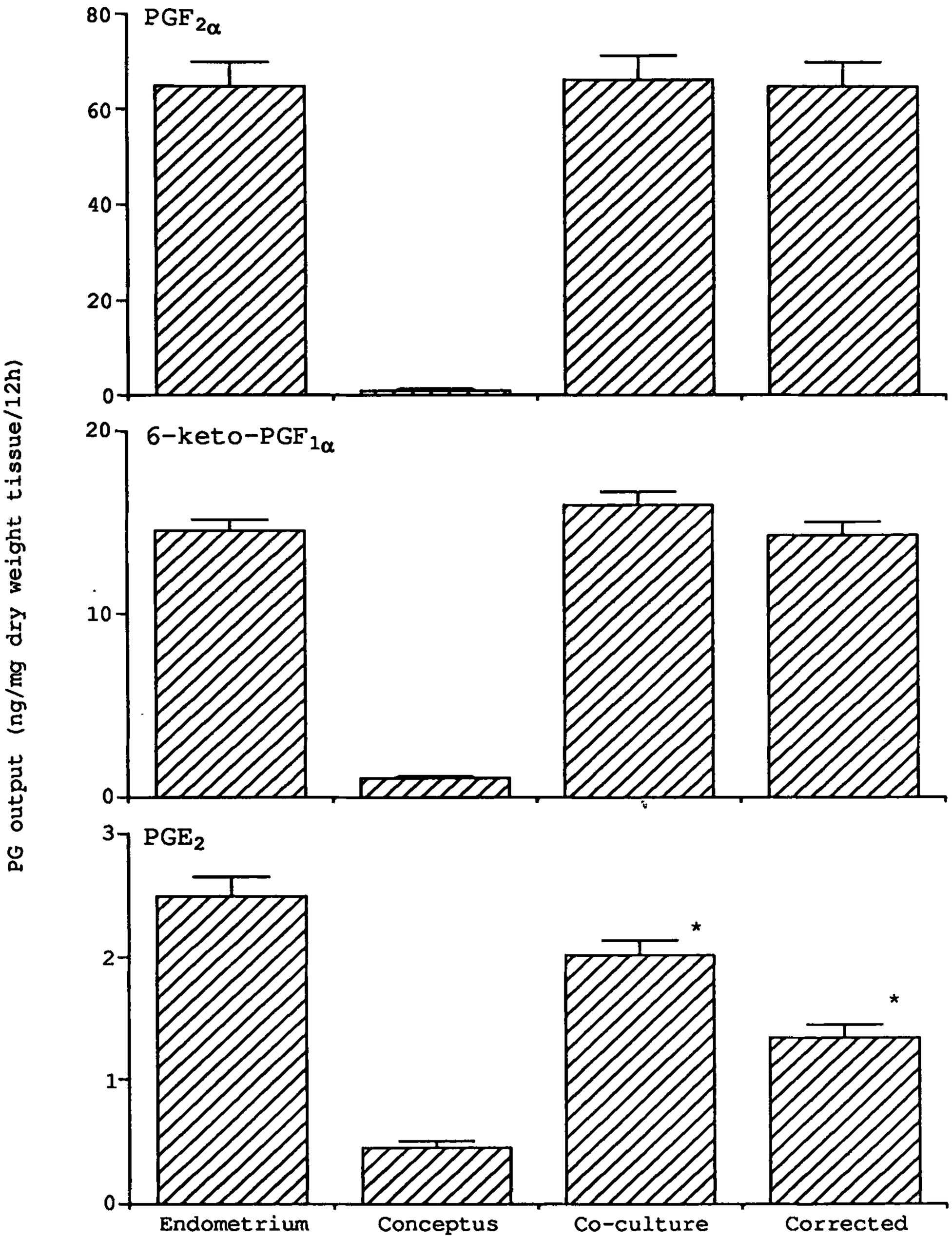


Fig 41. The outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium, Day-15 conceptus, and from Day-15 endometrium cultured (i) together with Day-15 conceptus (Co-culture) and (ii) after correcting for the amount of PGs produced by the Day-15 conceptus (Corrected), cultured for 12h. Results are mean ± s.e.m. (n = 15).*Significantly ($P < 0.05$) lower than the corresponding value for PG output from control endometrium for the same PG.

Conclusions

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium over two consecutive 6h periods of culture and of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium over a 12h period of culture were unaffected by co-culture with conceptus tissue from Day-15 pregnant guinea-pigs. Therefore, secretions from the Day-15 guinea-pig conceptus are unable to affect $PGF_{2\alpha}$ production by the Day-15 guinea-pig endometrium in culture. The output of PGE_2 from Day-15 guinea-pig endometrium was decreased by the presence of conceptus tissue over 12h of continuous culture compared to the output of PGE_2 from control Day-15 endometrium. However, the decrease was not large and may be due to an overestimation in correcting for conceptus PG production.

3:5:b The effect of co-culturing with Day-15 pregnant guinea-pig endometrium on the output of prostaglandins from Day-15 non-pregnant guinea-pig endometrium in culture

Introduction

Both oTP-1 and bCSP selectively stimulate the synthesis of proteins in the endometrium of cyclic sheep and cows, respectively (Godkin et al., 1984b; Vallet et al., 1987b; Salamonsen et al., 1988; Gross et al., 1988c; Helmer et al., 1989b). bCSP and bTP-1 induce an intracellular inhibitor of PGF synthesis and reduce secretion of PGF, but not PGE_2, from endometrial explants from Day-17 cyclic cows (Gross et al., 1988c; Helmer et al., 1989b). Thus the antiluteolytic mechanism in the cow may be mediated via conceptus secretory proteins inducing the synthesis of a protein in the endometrium which inhibits the production of $PGF_{2\alpha}$. To examine whether such a mechanism exists in the guinea-pig, the effect of co-culturing with Day-15 pregnant endometrium on the output of PGs by Day-15 cyclic endometrium in culture has been examined.

Methods

Five virgin female guinea-pigs which had undergone at least 3 normal oestrous cycles, were mated by placing with male guinea-pigs several days prior to oestrus. Guinea-pigs were examined daily for signs of mating and for the presence of sperm in the vaginal smear, with Day 1 of pregnancy being the first day of finding any of these signs.

The uteri were removed from five Day-15 cyclic and five Day-15 pregnant guinea-pigs. Under aseptic conditions, each uterine horn was "opened" via a longitudinal incision and the endometrium was

dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 18 petri dishes were prepared from the endometrial tissue from one cyclic and one pregnant Day-15 guinea-pig as follows: 6 dishes containing 5-10mg wet weight (1-2mg dry weight) of Day-15 cyclic endometrium (control cyclic endometrium), 6 petri dishes containing 5-10mg wet weight (1-2mg dry weight) of Day-15 pregnant endometrium (control pregnant endometrium), and 6 petri dishes containing 5-10mg wet weight (1-2mg dry weight) of Day-15 cyclic endometrium and 15-20mg wet weight (3-4mg dry weight) of Day-15 pregnant endometrium together (co-culture). In addition, 3 petri dishes were prepared each containing 50-60mg wet weight (10-12mg dry weight) of Day-15 pregnant endometrium.

9 petri dishes, comprising 3 petri dishes from each treatment group were incubated at 37°C for 12h. The other 9 petri dishes were incubated at 37°C for 6h at which time the medium from each dish was removed and replaced with fresh medium, except for the co-culture dishes where the medium was replaced by the medium from the 3 extra petri dishes containing Day-15 pregnant endometrium which had been cultured at 37°C for 6h. The 9 petri dishes were then incubated for a further 6h at 37°C.

The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each petri dish into preweighed containers and dried by placing in an oven at 37°C for 24h. The cyclic and pregnant endometrium from each of the co-culture dishes were removed and dried separately. Each container was then reweighed and the amount of dried endometrium from each petri dish was

calculated. The outputs of PGs were calculated per mg dry weight of cyclic or pregnant endometrium for dishes containing cyclic or pregnant endometrium alone respectively, and per mg dry weight of cyclic endometrium for the co-culture dishes. The amounts of PGs found in the co-culture dishes were also corrected for the production of PGs by the pregnant endometrium and, for the dishes which underwent replacement of medium at 6h, for the presence of PGs in the replacement medium, by using the data for PG output from pregnant endometrium alone.

Statistical tests

Changes in PG output with time and differences between PG outputs from Day-15 cyclic endometrium co-cultured with Day-15 pregnant endometrium and control Day-15 cyclic endometrium were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 cyclic guinea-pig endometrium significantly ($P < 0.05$) declined during 12h of culture. (Figure 42). The output of 6-keto-$PGF_{1\alpha}$ from Day-15 pregnant endometrium significantly ($P < 0.05$) declined whereas the outputs of $PGF_{2\alpha}$ and PGE_2 were unchanged during 12h of culture (Figure 42).

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 cyclic endometrium were significantly ($P < 0.01$) greater than the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 pregnant endometrium at every time period (Figures 42 and 43).

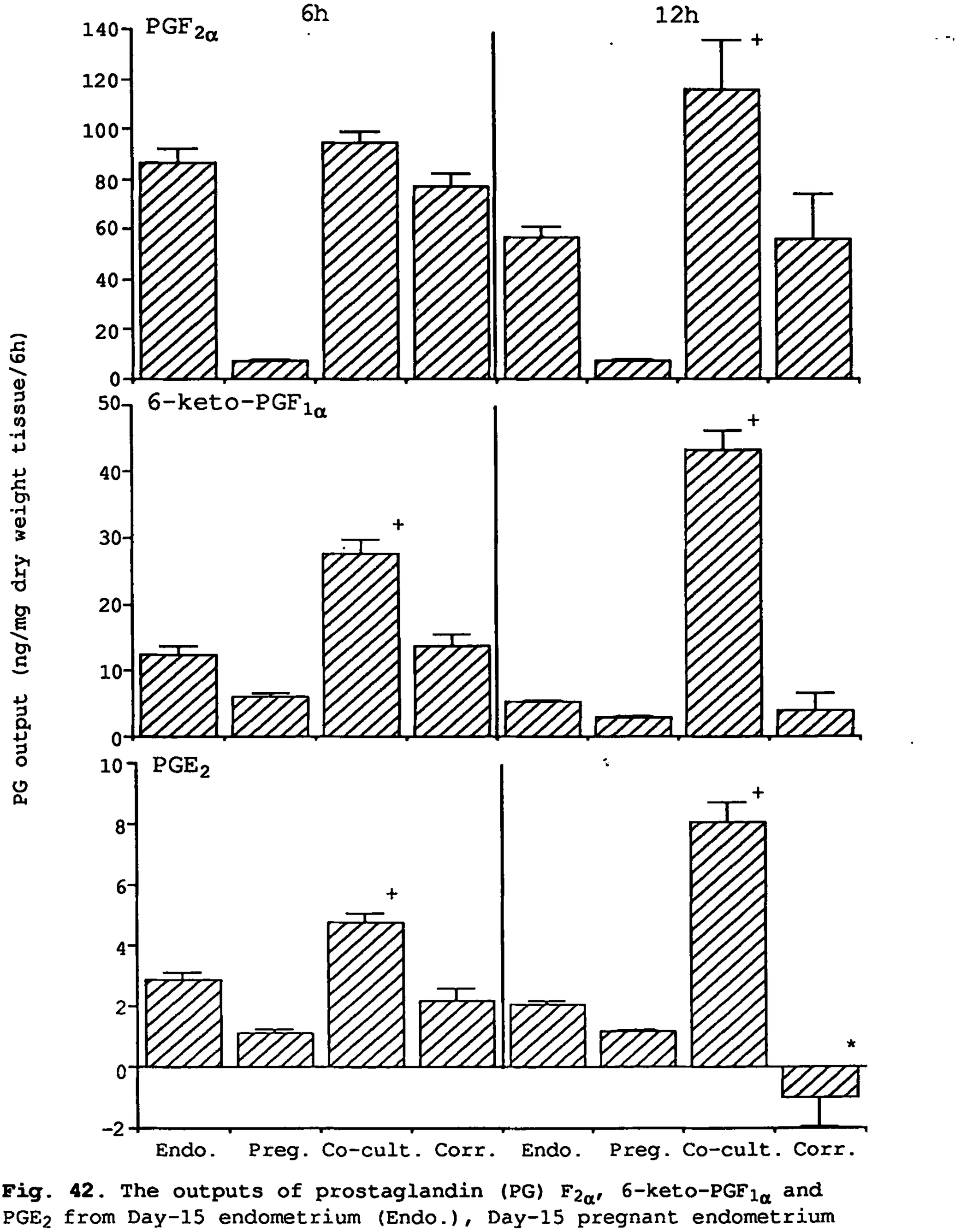


Fig. 42. The outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium (Endo.), Day-15 pregnant endometrium (Preg.), and from Day-15 endometrium cultured (i) together with Day-15 pregnant endometrium (Co-cult.) and (ii) after correcting (Corr.) for the amounts of PGs produced by the Day-15 pregnant endometrium, cultured for 12h with sampling at 6h. Results are mean ± s.e.m. (n = 15).*Significantly ($P < 0.05$) lower than the corresponding value for PG output from control endometrium for the same PG at the same time.+Significantly ($P < 0.05$) higher than the corresponding value for PG output from control endometrium for the same PG at the same time.

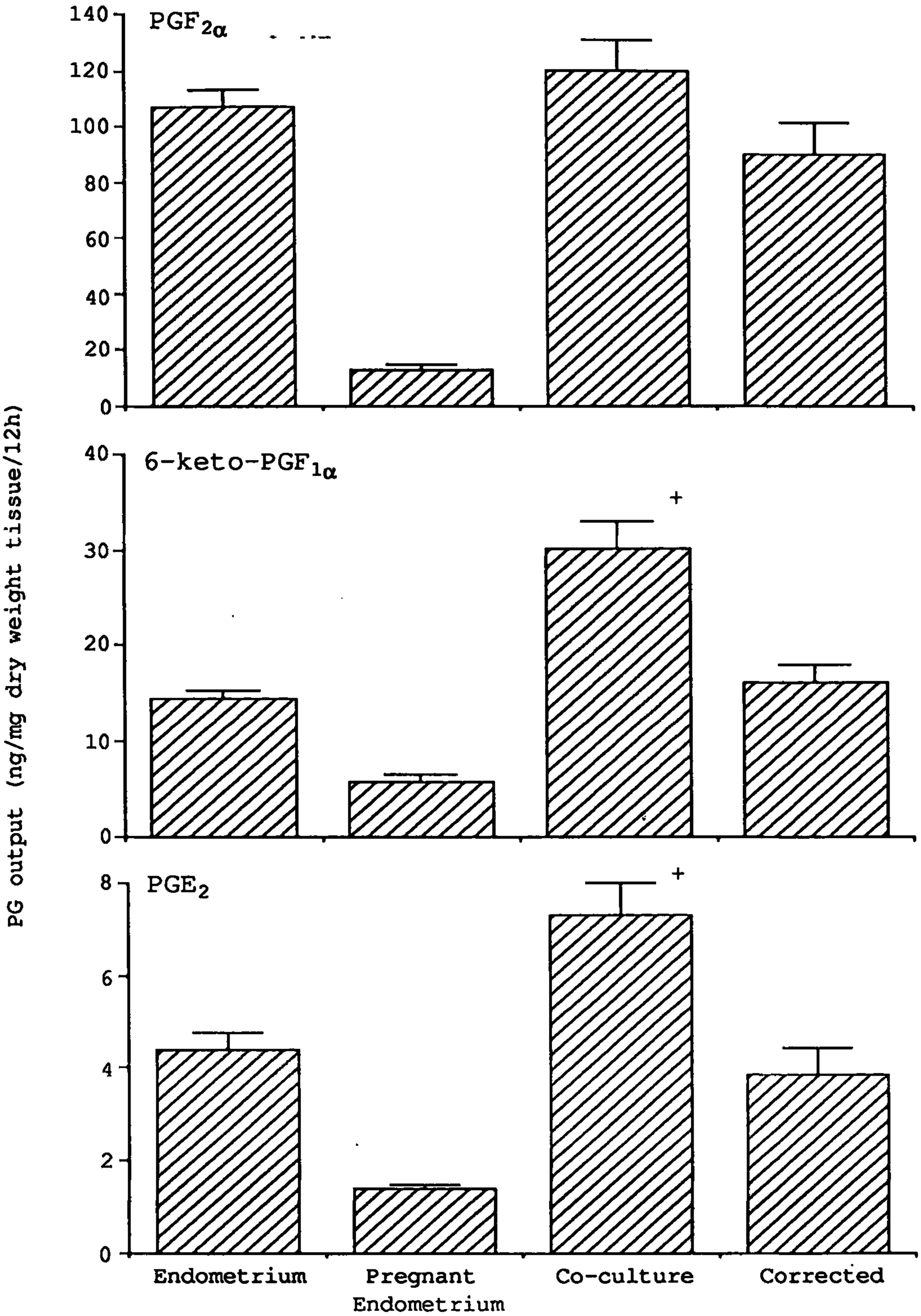


Fig. 43. The outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium, Day-15 pregnant endometrium and Day-15 endometrium cultured (i) together with Day-15 pregnant endometrium (Co-culture) and (ii) after correcting for the amounts of PGs produced by Day-15 pregnant endometrium (Corrected), cultured for 12h. Results are mean $\pm$ s.e.m. ($\underline{n}$ = 15). +Significantly ($\underline{P} < 0.05$) higher than the corresponding value for PG output from control endometrium for the same PG.

The output of $PGF_{2\alpha}$ from Day-15 cyclic endometrium co-cultured with Day-15 pregnant endometrium tended to increase after 6h compared to the output of $PGF_{2\alpha}$ from control Day-15 cyclic endometrium at this time and this increase was significant ($\underline{P} < 0.05$) after 12h of culture (Figure 42). However, after correction for $PGF_{2\alpha}$ output from Day-15 pregnant endometrium this increase was abolished (Figure 42). The outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 cyclic endometrium co-cultured with Day-15 pregnant endometrium were significantly ($\underline{P} < 0.05$) increased after 6h and 12h of culture compared to the outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from control Day-15 cyclic endometrium at these times (Figure 42). However, after correction for 6-keto-$PGF_{1\alpha}$ and PGE_2 output from Day-15 pregnant endometrium, these increases were abolished and the output of PGE_2 from Day-15 cyclic endometrium co-cultured with Day-15 pregnant endometrium was found to be significantly ($\underline{P} < 0.05$) decreased after 12h of culture compared to the output of PGE_2 from control Day-15 cyclic endometrium at this time (Figure 42).

The output of $PGF_{2\alpha}$ from Day-15 cyclic endometrium was unaffected by co-culturing with Day-15 pregnant endometrium after 12h of continuous culture compared to the output of $PGF_{2\alpha}$ from control Day-15 cyclic endometrium either before or after correction for $PGF_{2\alpha}$ production by Day-15 pregnant endometrium (Figure 43). The outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 cyclic endometrium were significantly ($\underline{P} < 0.05$) increased by co-culturing with Day-15 pregnant endometrium after 12h of continuous culture compared to the outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from control Day-15 cyclic endometrium (Figure 43). However, these increases were abolished after correction for 6-keto-$PGF_{1\alpha}$ and PGE_2 output from Day-15 pregnant endometrium (Figure 43).

Conclusions

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 during the first 6h period of culture were found to be 12.1-, 2.1- and 2.6-fold higher respectively from Day-15 cyclic endometrium than from Day-15 pregnant endometrium. Therefore PG output, and in particular $PGF_{2\alpha}$ output from the guinea-pig endometrium is suppressed during pregnancy.

The results presented here failed to demonstrate any effect of the Day-15 pregnant guinea-pig endometrium on the output of PGs from the Day-15 cyclic guinea-pig endometrium. There was a significant decrease in the output of PGE_2 from Day-15 cyclic endometrium co-cultured with Day-15 pregnant endometrium as compared to the output of PGE_2 from control Day-15 cyclic endometrium between 6h and 12h of culture after correction for PGE_2 output by the Day-15 pregnant endometrium. However, this was probably due to the difficulty in approximating the amount of PGE_2 added to the culture medium when the original medium was replaced by medium from dishes in which Day-15 pregnant endometrium had been cultured for 6h. As the outputs of PGE_2 from the Day-15 cyclic and the Day-15 pregnant endometrium are both low at this time and there is only a two-fold difference between them any overestimate of the amount of PGE_2 in the replacement medium might have caused this result. Medium from Day-15 pregnant endometrium cultured for 6h was therefore also without effect on the output of PGs (especially of $PGF_{2\alpha}$) from Day-15 cyclic guinea-pig endometrium.

DISCUSSION

The output of PGs, and in particular $PGF_{2\alpha}$, were found to be significantly higher from cyclic than from pregnant endometrium in

culture on Day-15. These findings agree with those of previous studies in which the synthesis of $PGF_{2\alpha}$ was found to be significantly less by uterine homogenates of Day-15 bilaterally pregnant guinea-pigs when compared to Day-15 cyclic guinea-pigs (Maule Walker and Poyser, 1973, 1974). In addition, the levels of $PGF_{2\alpha}$ measured in the utero-ovarian vein of pregnant guinea-pigs were found to be lower than those in cyclic guinea-pigs on Day 15 (Blatchley et al., 1975a, 1975b; Antonini et al., 1976), and the $PGF_{2\alpha}$ output from the guinea-pig uterus superfused in vitro was much lower on Day 15 of pregnancy than on Day 15 of the cycle (Poyser, 1984a). Thus, the synthesis of $PGF_{2\alpha}$ by the guinea-pig uterus is specifically inhibited during pregnancy.

The mechanism by which the guinea-pig conceptus mediates its antiluteolytic mechanism is unknown. Protein synthesis inhibitors significantly inhibit $PGF_{2\alpha}$ production by Day-15 non-pregnant guinea-pig endometrium in culture (Riley and Poyser, 1989), and significantly ($P < 0.05$) more [^{3}H]-leucine is incorporated into secreted guinea-pig endometrial proteins on Day 15 of the cycle than on Day 7 of the cycle and on Day 15 of pregnancy (Abdi-Dezfuli and Poyser, 1989). Therefore, the guinea-pig conceptus may exert its antiluteolytic effect by inhibiting the synthesis of an oestradiol-stimulated protein; this protein has been proposed to act on a progesterone-primed uterus to raise the endometrial intracellular free calcium concentration via an influx of extracellular calcium and/or release of intracellular calcium and thereby stimulating endometrial $PGF_{2\alpha}$ synthesis (Poyser, 1984a). However, protein synthesis inhibitors significantly inhibit $PGF_{2\alpha}$ production from Day-15 non-pregnant endometrium in culture within 6h (Riley and Poyser, 1989), whereas the presence of Day-15 conceptus

tissue had no effect on endometrial $PGF_{2\alpha}$ production after 6h and even after 12h. Consequently, it appears that, if the guinea-pig conceptus does prevent endometrial $PGF_{2\alpha}$ synthesis by an inhibitory action on protein synthesis, insufficient amounts of a "protein synthesis inhibiting factor" are produced by the guinea-pig conceptus in culture to have any effect on the cultured Day-15 endometrium from non-pregnant guinea-pigs.

PGE_2 was previously suggested to be the antiluteolytic factor in sheep when PGs of the E series were shown to lessen the luteolytic effects of $PGF_{2\alpha}$ when given concomitantly (Henderson, Scaramuzzi and Baird, 1977; Mapletoft, Miller and Ginther, 1977; Reynolds, Stigler, Hoyer, Magness, Huie, Huecksteadt, Whysong, Behrman and Weems, 1981). In addition, both PGE_1 and PGE_2 have been shown to stimulate progesterone production in bovine luteal tissue (Speroff and Ramwell, 1970; Sellner and Wickersham, 1970; Marsh, 1971). However, PGI_2 is 100-1000 times more potent than PGE_2 in inducing corticosteroidogenesis in dispersed cat adrenocortical cells (Ellis, Sten, Schrey, Carchman and Rubin, 1978), and injections of PGI_2 into the bovine corpus luteum significantly increased peripheral plasma progesterone whereas 6-keto-$PGF_{1\alpha}$ was without effect (Milvae and Hansel, 1980b). PGI_2 also increased progesterone accumulation in dispersed luteal cells (Milvae and Hansel, 1980) and thus PGI_2 has a luteotrophic effect on the bovine corpus luteum both in vivo and in vitro. These results led some authors to consider that PGE_2 or PGI_2 could have an important role in the maintenance of luteal structures during early pregnancy. Indeed, an enhancement in PGE_2 synthesis by the pregnant sheep endometrium, which occurs simultaneously with an increase in PGE_2 concentration in utero-ovarian venous plasma, has

been demonstrated by several authors (Ellinwood, Nett and Niswender, 1979; Lacroix and Kann, 1982; Silvia, Ottobre and Inskeep, 1984). However, there is no increase in the concentration of 6-keto-$PGF_{1\alpha}$ in utero-ovarian plasma of early pregnant sheep (Silvia et al., 1984), and thus there is no evidence that a change in the secretion of PGI_2 is involved in maintenance of the corpus luteum of pregnancy in the sheep. The experiments in this thesis have shown that neither PGE_2 nor PGI_2 from either the guinea-pig conceptus or endometrium are likely to be the antiluteolytic factors in the guinea-pig, as production of PGs from Day-15 conceptuses was very low and the outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 pregnant guinea-pig endometrium were significantly less than from Day-15 cyclic guinea-pig endometrium at every time period studied. In addition, neither the concentration of PGE_2 in the utero-ovarian vein of the guinea-pig (Antonini et al., 1976) nor the outputs of PGE_2 and 6-keto-$PGF_{1\alpha}$ from the superfused guinea-pig uterus (Poyser, 1984a) are increased during early pregnancy. In the present study, the release of PGE_2 from Day-15 non-pregnant endometrium was significantly inhibited over a 12h period of culture with conceptus tissue. This may have been due to metabolism of PGE_2 by the conceptus. However although previous experiments (Maule Walker and Poyser, 1978) showed that the metabolism of PGE_2 by homogenates of Day-15 conceptus was higher than the metabolism of $PGF_{2\alpha}$ by homogenates of Day-15 conceptus, the metabolism of both PGs was low (14-22%) and insufficient to explain the extent of the reduction in release of $PGF_{2\alpha}$ from the Day-15 pregnant guinea-pig uterus (Poyser, 1984a). In addition, these metabolism experiments were carried out in the presence of NAD^+, and in the absence of NAD^+ there was no detectable metabolism of $PGF_{2\alpha}$ by uterine

homogenates compared to about 18% metabolism of $PGF_{2\alpha}$ by uterine homogenates in the presence of NAD^+ (Poyser, 1979). Therefore, in the above experiments which were carried out in the absence of NAD^+, metabolism of PGs by the conceptus was likely to be very low.

Factors secreted by Day-15 pregnant guinea-pig endometrium were unable to affect PG production by Day-15 cyclic guinea-pig endometrium. This is in conflict with results from the cow where endometrial intracellular preparations from pregnant cows markedly decreased PGF synthesis by cotyledonary microsomes from parturient cows (Gross et al., 1988b), and synthesis of PGF by the cotyledonary PG-generating system was decreased when incubated with the cytosol from Day-16 bovine endometrial explants treated with bCSP (Gross et al., 1988c) or bTP-1 (Helmer et al., 1989b). As the Day-15 pregnant guinea-pig endometrium used in these experiments was removed from the guinea-pig immediately prior to culture and would have been exposed to any conceptus protein secretions in vivo it was presumed that any induced endometrial intracellular inhibitor of PGF synthesis would already be present. However, the intracellular inhibitor of PGF synthesis in the cow endometrium is found in the cytosol and any similar inhibitor present in the pregnant guinea-pig endometrium might not have been secreted into the culture medium and therefore would not have been able to affect PG synthesis in other tissues e.g. the Day-15 cyclic guinea-pig endometrium.

Overall, co-culturing the conceptus or Day-15 pregnant endometrium with Day-15 non-pregnant endometrium failed to inhibit the high output of $PGF_{2\alpha}$ from the Day-15 non-pregnant endometrium. It is possible that the conceptus was secreting proteins which could inhibit endometrial $PGF_{2\alpha}$ production, but the amounts released

were too small to reach a sufficient concentration in the culture medium to have an inhibitory effect. Thus it was decided to isolate and purify proteins secreted by the guinea-pig conceptus so that a higher concentration could be tested on the Day-15 non-pregnant endometrium.

3:6 INVESTIGATIONS INTO THE CHARACTER AND ACTIVITIES OF PROTEINS SECRETED BY THE GUINEA-PIG CONCEPTUS

3:6:a Isolation of proteins secreted by the Day-15 guinea-pig conceptus in culture

Introduction

Both ovine and bovine conceptus secretory proteins (oCSP and bCSP) have been shown to extend the lifespan of the corpus luteum when infused into the uterine lumen of the cyclic sheep and cow respectively (Godkin et al., 1984a; Knickerbocker et al., 1986a). In addition, bCSP reduces $PGF_{2\alpha}$ levels in the vena cava and attenuates uterine $PGF_{2\alpha}$ production in response to oestradiol when infused intrauterinely into cyclic cows (Knickerbocker et al., 1986b). Therefore, proteins secreted by the conceptus are responsible for the prevention of luteal regression during pregnancy in the sheep and cow. The specific proteins responsible for extending the lifespan of the corpus luteum of pregnancy in these species have been isolated (ovine and bovine trophoblast protein-1; oTP-1 and bTP-1) and have been found to be low molecular weight, acidic proteins which share significant structural and functional homology with α-interferon.

However, studies in this thesis have shown that the guinea-pig conceptus does not secrete any antiviral activity indicative of interferon (see Section 3:4:b) and human α-interferon does not inhibit the output of PGs from the Day-15 guinea-pig endometrium in culture (see Section 3:4:a). These results suggest that the antiluteolytic effect of the guinea-pig conceptus (Bland and Donovan, 1969b; Poyser, 1984a) is not due to the secretion of proteins which are similar to oTP-1 and bTP-1.

To examine whether proteins secreted by the conceptus are responsible for the prevention of luteal regression in the guinea-pig, culture medium obtained from Day-15 conceptuses was purified by a series of chromatographic techniques in order to isolate any proteins secreted into the medium. The proteins found were examined for their effects on the output of PGs from the Day-15 non-pregnant guinea-pig endometrium and on the activity of PLA_2.

Methods

Female guinea-pigs which had exhibited at least two normal oestrous cycles were mated by placing with males several days before oestrus. Day 1 of pregnancy was taken as the first day on which sperm was present in the vaginal smear and/or signs of mating were found.

The uteri were removed from thirty-one Day-15 pregnant guinea-pigs. Under aseptic conditions, each uterine horn was "opened" by a longitudinal incision and the conceptuses were gently removed. Each conceptus was cut into 1-2mm^3 pieces and 8 petri dishes were prepared from the conceptus tissue from each guinea-pig (approximately 60-100mg dry weight). The petri dishes were incubated at 37°C for 24h. The culture medium was removed from each dish and pooled before undergoing dialysis, desalting and lyophylization (see Section 2:10). The dried material obtained was weighed and analysed for protein content by Lowry assay. After culture, the conceptus tissue from each dish was removed and amalgamated into a preweighed container. The tissue was dried by heating in an oven at 37°C for 24h. The container was reweighed and the amount of dried conceptus tissue was calculated. The molecular weights of the proteins isolated from the culture medium of the Day-15 guinea-pig

conceptus were determined by SDS PAGE (see Section 2:8).

Results

A total of 3.44g of dried conceptus tissue was obtained from 31 Day-15 pregnant guinea-pigs. After dialysis, desalting and lyophylization a total of 1.02g of dried material was obtained from the culture medium of the conceptus tissue. The protein content of the conceptus tissue as determined by Lowry assay was found to be 80-90%.

The molecular weight profile on SDS PAGE of the proteins isolated from the conceptus culture medium is shown in Figure 44. A heavily stained band which co-migrated with the molecular weight marker for bovine serum albumin (67kDa) was found at 68.3kDa. Other stained bands were found at 97.4kDa, 76.5kDa and 74.6kDa. A more lightly stained band was detected at 15.1kDa.

Conclusions

3.44mg of dried conceptus tissue released approximately 816-918mg of protein into the culture medium in 24h. Thus, the conceptus tissue secreted 23.7-26.7% of its own dried weight as protein in this time.

The presence of proteins at 74.6-76.5kDa and 68.3kDa suggests that there is contamination of guinea-pig conceptus secretory protein with the serum proteins transferrin (76.5kDa) and albumin (67.0kDa). In order to remove the serum protein contaminant albumin from guinea-pig conceptus secretory protein, it was decided to purify the secreted protein by affinity chromatography on Blue Sepharose CL-6B.

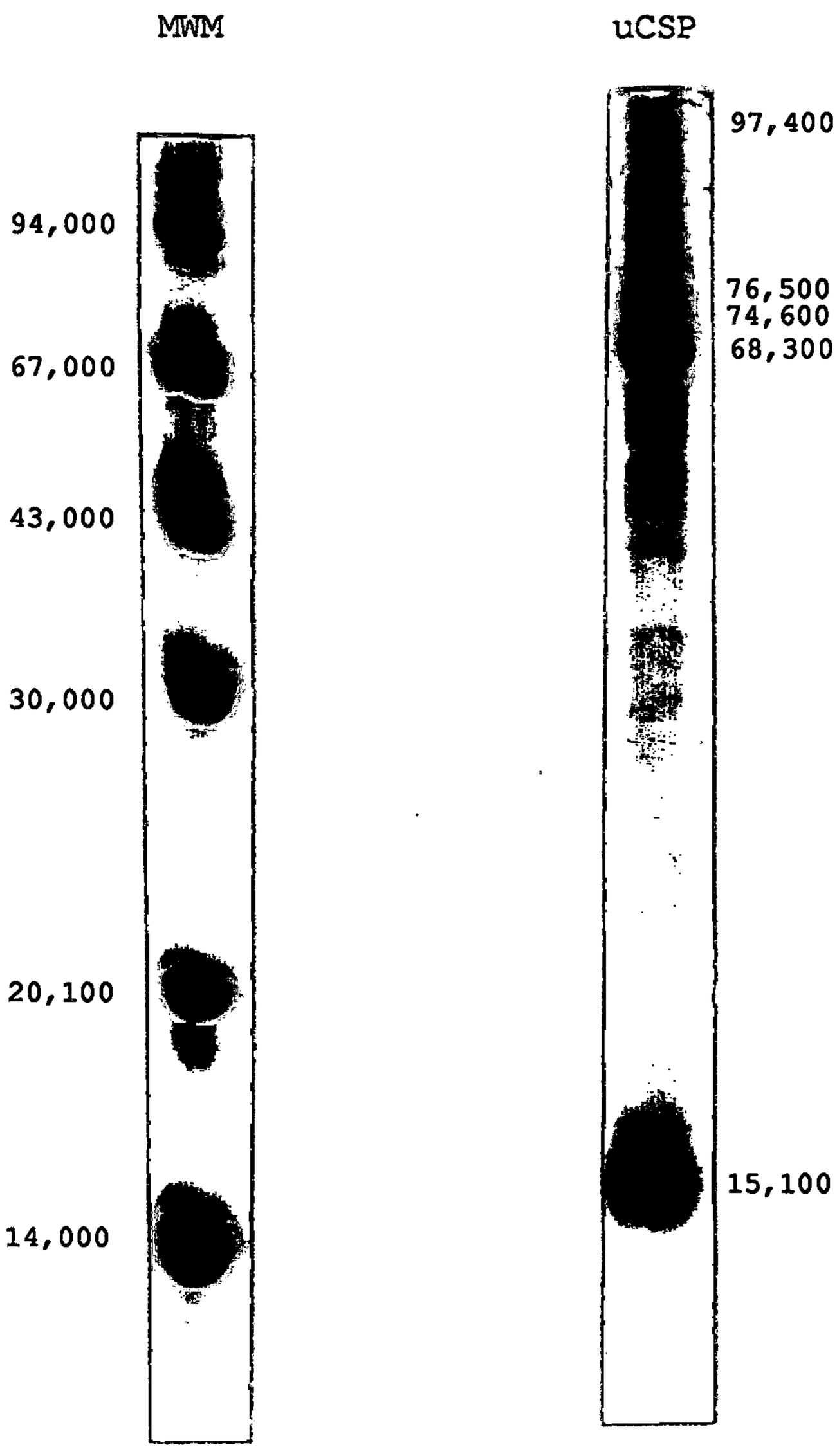


Fig. 44. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profile of unpurified conceptus secretory proteins (uCSP) from the Day-15 pregnant guinea-pig. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of uCSP were run on the gel.

3:6:b Purification of guinea-pig-conceptus secretory proteins by affinity chromatography

Introduction

Analysis of the proteins purified from Day-15 guinea-pig conceptus culture medium by SDS PAGE revealed the presense of large amounts of serum albumin. In order to remove the albumin contamination, the conceptus secretory proteins were subjected to purification on Blue Sepharose CL-6B.

Blue Sepharose CL-6B consists of the dye Cibacron Blue F3 G-A covalently attached to the cross-linked agarose gel Sepharose CL-6B. Cibacron Blue F3 G-a binds to a wide variety of enzymes and proteins including albumin (Travis et al., 1976). Proteins from human uterine flushings were successfully depleted of albumin by affinity chromatography on Blue Sepharose CL-6B (MacLaughlin and Richardson, 1983). However, Cibacron Blue F3 G-a also binds interferon (Jankowski et al., 1976) to which the antiluteolytic proteins secreted by the embryos of the sheep (Godkin et al., 1984a) and cow (Helmer et al., 1989a) have been shown to share structural and functional homology. Therefore, the proteins which bound to the Blue Sepharose column were retained in order to study their effects on PG synthesis as well as those proteins which eluted directly from the Blue Sepharose column.

Methods

A total of 946.4mg of dried protein from the culture medium of Day-15 guinea-pig conceptuses was purified on Blue Sepharose in batches. Each batch of protein was dissolved in 2ml of Sample Buffer to a concentration which did not excede 50mg/ml (at 70mg/ml the

protein solution becomes too viscous to run uniformly through the column). The procedures used for carrying out affinity chromatography on Blue Sepharose CL-6B are described in Section 2:10:(i). The proteins which eluted directly from the column (PBS) and the proteins which were retained on Blue Sepharose until eluted with high salt buffer (ROBS) were collected into two fractions and were dialysed, desalted and lyophylized (see Section 2:10) before being weighed. The molecular weights of the proteins present in the PBS and ROBS fractions were determined by SDS PAGE (see Section 2:8).

Results

A total of 660.6mg of protein was recovered from the 946.4mg of protein applied to the Blue Sepharose column (69.8%). Of the recovered protein, 550.9mg (83.4%) were found in the fraction which eluted directly from the Blue Sepharose column (PBS) and 109.7mg (16.6%) were retained on the Blue Sepharose column until eluted with high salt buffer (ROBS).

The molecular weight profiles of the PBS and ROBS fractions on SDS PAGE are shown in Figure 45. Both fractions contained similar protein bands except that the ROBS fraction contained neither the 97.4kDa nor the 74.6kDa proteins found in the PBS fraction (Figure 45).

Conclusions

Affinity chromatography on Blue Sepharose CL-6B did not succeed in removing all of the albumin from the PBS fraction as the band corresponding to the molecular weight marker for bovine serum albumin (67kDa) was present in the SDS PAGE profile of the PBS

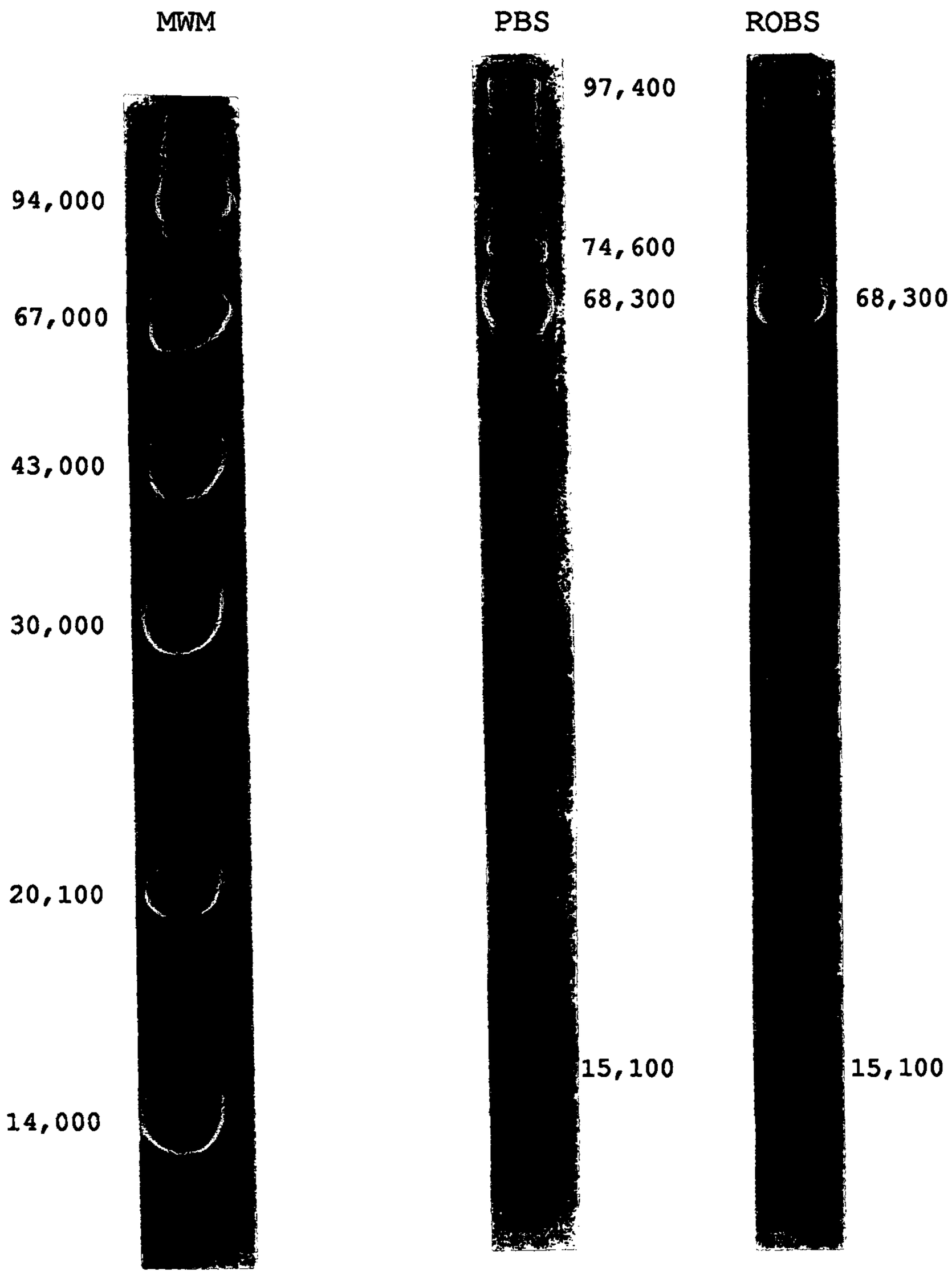


Fig. 45. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of conceptus secretory proteins from the Day-15 pregnant guinea-pig (i) which had no affinity for Blue Sepharose (PBS) and (ii) which were retained on Blue Sepharose (ROBS). The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

fraction. Albumin binds to Cibacron Blue F3 G-A in a less-specific manner than some other proteins and it may have been that the ionic strength or the pH of the buffer was not ideal for the maximal binding of albumin to the dye. Alternatively the concentration of albumin in uCSP may have exceded the binding capacity of the column.

However affinity chromatography on Blue Sepharose CL-6B did result in the resolution of the 97.4kDa and the 74.6kDa proteins into the PBS fraction. This result provides further evidence that the 74.6kDa protein is transferrin as transferrin shows no affinity for Blue Sepharose, and Blue Sepharose is used to purify commercially produced transferrin which contains albumin as a contaminant (Pharmacia AB, Uppsala, Sweden).

3:6:c Purification of guinea-pig conceptus-secretory proteins by ion-exchange chromatography

Introduction

In order to identify and purify further the proteins obtained from the culture medium from Day-15 guinea-pig conceptuses, the PBS fraction was subjected to separation by ion-exchange chromatography. Separation of molecules by ion-exchange chromatography is achieved on the basis of the charge carried by each molecule. Proteins are eluted from the column by varying either the ionic strength or the pH of the buffer thus changing the affinity of the molecule for the ion-exchanger. The anionic exchanger diethylaminoethyl (DEAE) linked to Sepharose CL-6B was used in conjunction with the Pharmacia Gradient Mixer GM-1 in order to obtain elution of proteins with an ionic gradient. Ion exchange chromatography is able to resolve extremely complex mixtures of proteins as proteins which have closely similar molecular weights may be separated on the basis of very small differences in charge.

Methods

411.1mg of the PBS fraction were separated on the ion-exchange gel DEAE Sepharose CL-6B. The protein was run in several batches at a concentration of not more than 30mg/ml. The procedures used for the separation of proteins by ion-exchange chromatography are described in Section 2:10:(ii). An ionic gradient from 0-0.5M NaCl was used to elute the proteins which were collected in 5ml volumes on a Fraction Collector. The proteins were resolved into 6 fractions on the basis of the peaks of absorbance observed on the chart recording of the elution profile. Each fraction was dialysed, desalted and

lyophylized (see Section 2:10) before being weighed. The molecular weights of the proteins in each fraction were determined by SDS PAGE (see Section 2:8).

Results

A total of 178.4mg of the 411.1mg of the PBS fraction applied to the ion-exchange column were recovered (43.4%). The elution profile of the PBS fraction from the ion-exchange column is shown in Figure 46. The 6 peaks were eluted at ionic strengths of NaCl of 0.1M (F1), 0.175M (F2), 0.2M (F3), 0.225M (F4), 0.3M (F5) and 0.35M (F6). The total protein recovered in each fraction was 3.3mg, 13.5mg, 41.6mg, 73.7mg, 34.2mg and 12.1mg in F1, F2, F3, F4, F5 and F6, respectively.

The molecular weight profiles obtained from SDS PAGE analysis of F1-F6 are shown in Figure 47. F1 contained bands at 97.4kDa, 68.3kDa, 29.6kDa and 17.6kDa (Figure 47). F2 contained a 97.4kDa protein as well as four discrete bands of similar molecular weight to each other at 74.6kDa, 71.0kDa, 68.3kDa and 65.7kDa. Further bands were also seen at 38.2kDa and 31.3kDa with a diffuse band from approximately 20.0kDa down to a maximum intensity of staining at 15.1kDa (Figure 47). F3 contained several high molecular weight proteins at 97.4kDa, 96.5kDa and 94.6kDa with heavily stained bands at 74.6kDa and 68.3kDa. Further bands were found at 38.2kDa and 35.7kDa with an intensely stained diffuse band at 15.1kDa (Figure 47). F4 contained bands at 96.5kDa, 94.6kDa and 74.6kDa with a heavily stained band at 68.3kDa and light staining at 15.1kDa (Figure 47). F5 contained a range of bands from 97.4-29.6kDa with further staining at 15.1kDa (Figure 47). F6 contained bands at 97.4kDa and 74.6kDa, a heavily stained band at 68.3kDa and a

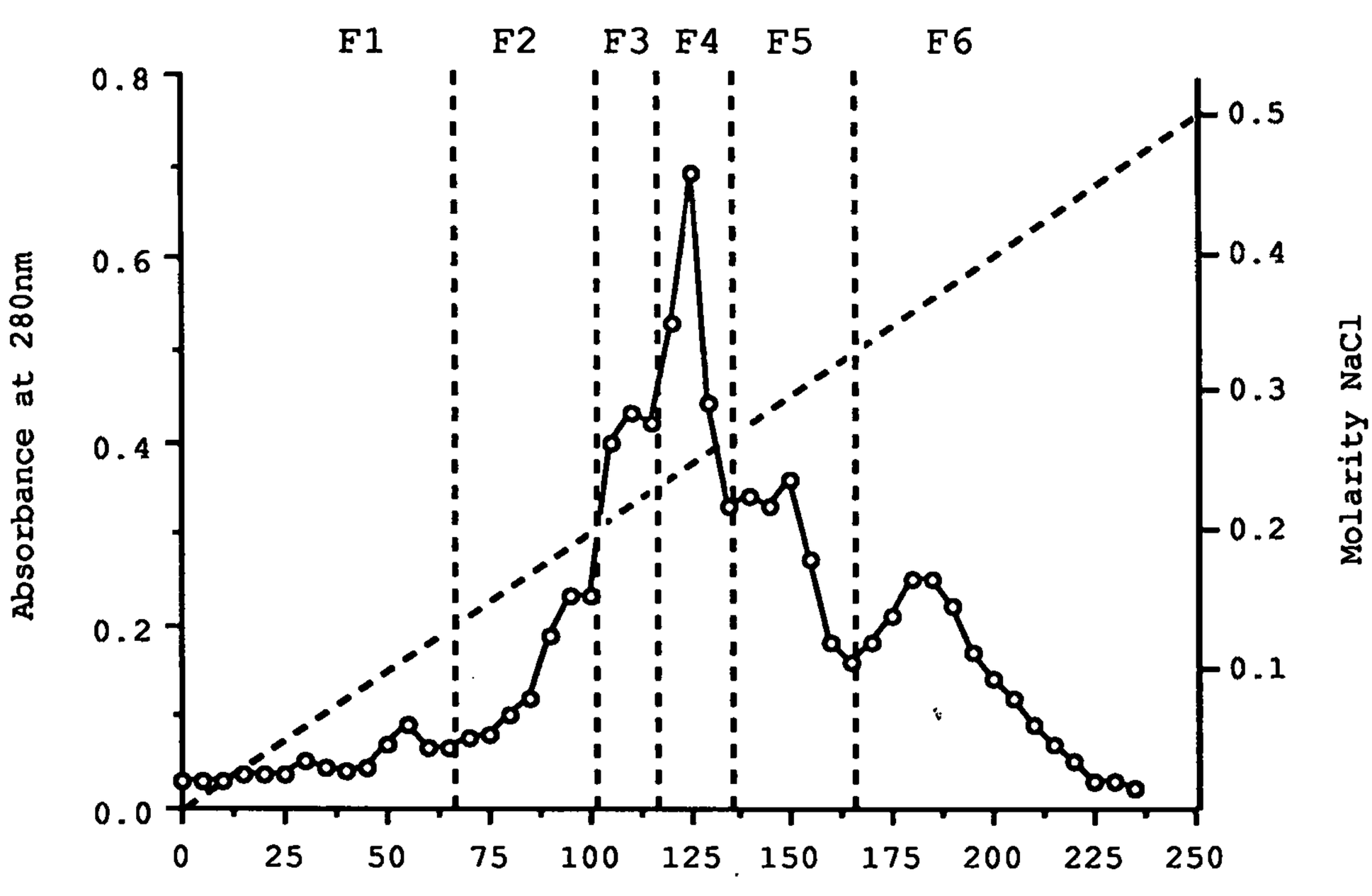


Fig. 46. The elution profile of guinea-pig conceptus secretory proteins, which were not retained on Blue Sepharose (PBS; 37.32mg/ml), from an ion-exchange column (DEAE Sepharose CL-6B) with an ionic gradient of 0-0.5M NaCl. The proteins were collected on a Fraction Collector in 5ml volumes and separated into 6 fractions (F1-F6) on the basis of the peaks of absorbance observed on the elution profile.

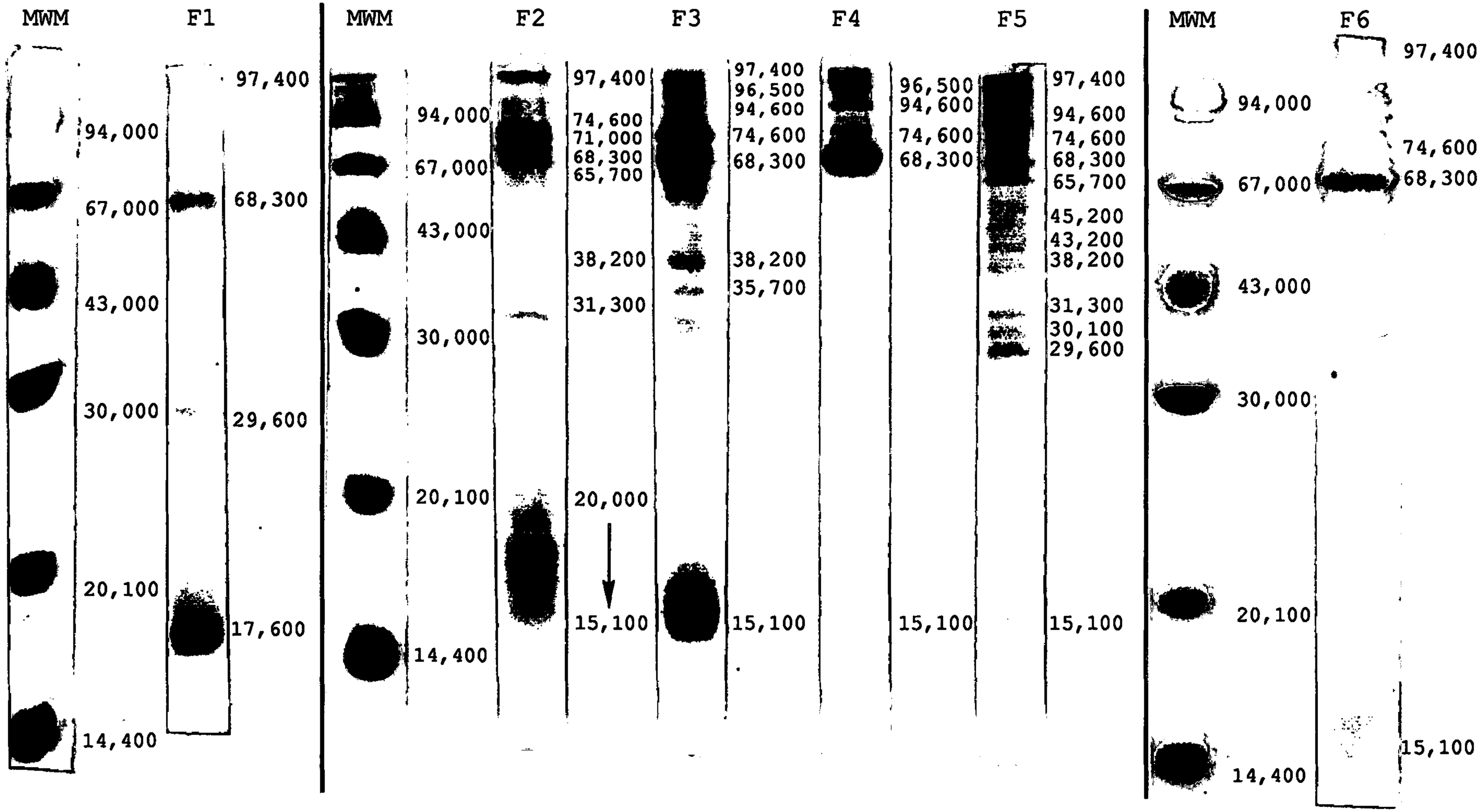


Fig. 47. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of F1-F6 of guinea-pig conceptus secretory proteins from the PBS fraction separated by ion-exchange chromatography. F1 and F6 were run on separate gels from F2, F3, F4 and F5 and the SDS PAGE profiles of molecular weight markers (MWM) run on each gel are shown for comparison. 10µg of each protein fraction were run on the gels.

diffusely stained band around 15.1kDa (Figure-47).

Conclusions

The majority of the albumin in guinea-pig conceptus secretory protein was resolved into F3 and F4 by ion-exchange chromatography. These fractions eluted at an ionic strength of 0.2-0.225M NaCl. A similar ionic strength of NaCl has been shown to elute albumin when it is being separated from haemoglobin on DEAE Sephadex A-50 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The majority of the protein band corresponding to transferrin was eluted in F3 at an ionic strength of 0.2M NaCl. F2 exhibited 4 discrete bands of protein in the region of transferrin and albumin suggesting that other proteins of similar molecular weight are present which were previously obscured by the width of the transferrin and albumin bands. In addition all of the fractions contained low molecular weight proteins around 15.1-17.6kDa. In F2 and F6, the 15.1kDa protein staining was very diffuse and included a region up to about 17.6kDa. The 15.1kDa protein was most heavily stained in F3.

3:6:d The effects of guinea-pig conceptus secretory proteins, separated by ion-exchange chromatography, on the output of prostaglandins from Day-15 guinea-pig endometrium in culture

Introduction

bCSP reduces $PGF_{2\alpha}$ levels in the vena cava and attenuates $PGF_{2\alpha}$ production in response to oestradiol when infused into the uterine lumen of cyclic cows (Knickerbocker et al., 1986b). Purified bTP-1 also reduces $PGF_{2\alpha}$ concentrations in the vena cava when infused intrauterinely into cows (Helmer et al., 1989a). oTP-1 attenuates the release of $PGF_{2\alpha}$ and PGE from cultured ovine endometrial cells (Salamonsen et al., 1988). Therefore, proteins secreted by the sheep and cow conceptus can inhibit uterine $PGF_{2\alpha}$ production. $PGF_{2\alpha}$ concentrations in the utero-ovarian vein of pregnant guinea-pigs are low on Day-15 compared to the concentrations found in the utero-ovarian vein of the cyclic guinea-pig on Day-15 (Blatchley et al., 1975a, 1975b; Antonini et al., 1976). In addition, it has been shown in these studies (see Section 3:5:b) that $PGF_{2\alpha}$ production by guinea-pig endometrium in culture is much lower on Day-15 of pregnancy than on Day-15 of the cycle. To examine whether proteins secreted by the guinea-pig endometrium are responsible for the inhibition of endometrial $PGF_{2\alpha}$ synthesis in the pregnant guinea-pig, the effects of F1-F6 were examined on the outputs of PGs from Day-15 non-pregnant guinea-pig endometrium in culture.

Day-15 is a day of high PG, particularly of $PGF_{2\alpha}$, production by the guinea-pig endometrium and several compounds including indomethacin, protein synthesis inhibitors, calmodulin antagonists,

TMB-8, EGTA and lack of intracellular calcium have been shown to inhibit PG output from the Day-15 guinea-pig endometrium in culture (Riley and Poyser, 1987a, 1987b, 1989). Therefore, the culture system is sensitive to changes in PG output by Day-15 endometrium caused by a variety of mechanisms and it was assumed that this system would be able to detect any changes in PG output from Day-15 endometrium caused by guinea-pig conceptus secretory proteins.

Methods

The uteri were removed from five Day-15 non-pregnant guinea-pigs. Under aseptic conditions, each uterine horn was opened via a longitudinal incision and the endometrium was dissected away from the myometrium. The endometrium was cut into 1-2mm^3 pieces and 14 petri dishes were prepared containing 5-10mg wet weight (1-2mg dry weight) of endometrium from each uterus. Pairs of dishes were treated with 25μg/ml each of F1, F2, F3, F4, F5, F6 and no protein (control). The petri dishes were incubated at 37°C for 12h and the culture medium was removed and replaced with fresh medium containing the same protein treatments at 6h. The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture the pieces of endometrium were removed from each dish into separate preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was reweighed and the amount of dried endometrium from each dish was calculated. PG output was calculated per mg dry weight of endometrium.

Statistical tests

Changes in the output of PGs with time and differences between

treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by variance ratio F test, by a modified t test for unequal variances.

Results

The control outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-15 endometrium significantly ($P < 0.05$) declined during 12h of culture (Figure 48). The output of $PGF_{2\alpha}$ did not significantly change during 12h of culture.

None of the protein fractions F1-F6 (25μg/ml) affected the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium during the first 6h period of culture or the outputs of $PGF_{2\alpha}$ of PGE_2 at 12h of culture (Figure 48). However, F1 and F5 significantly ($P < 0.05$) stimulated the output of 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium in culture at 12h (Figure 48).

Conclusions

None of the protein fractions F1-F6, which were purified from guinea-pig conceptus culture medium by ion-exchange chromatography, inhibited the output of PGs from Day-15 guinea-pig endometrium in culture over 12h. It would appear that the proteins in these fractions are not responsible for the reduction in endometrial $PGF_{2\alpha}$ synthesis in the pregnant guinea-pig.

The stimulation of 6-keto-$PGF_{1\alpha}$ output, but not of $PGF_{2\alpha}$ or PGE_2 outputs, by F1 and F5 suggests that endometrial PGI_2 synthesis is controlled differently than $PGF_{2\alpha}$ or PGE_2 synthesis. However the increase in 6-keto-$PGF_{1\alpha}$ output was not large and may not have been due to an effect on endometrial PLA_2. It was therefore decided to examine the effects of F1-F6 on the

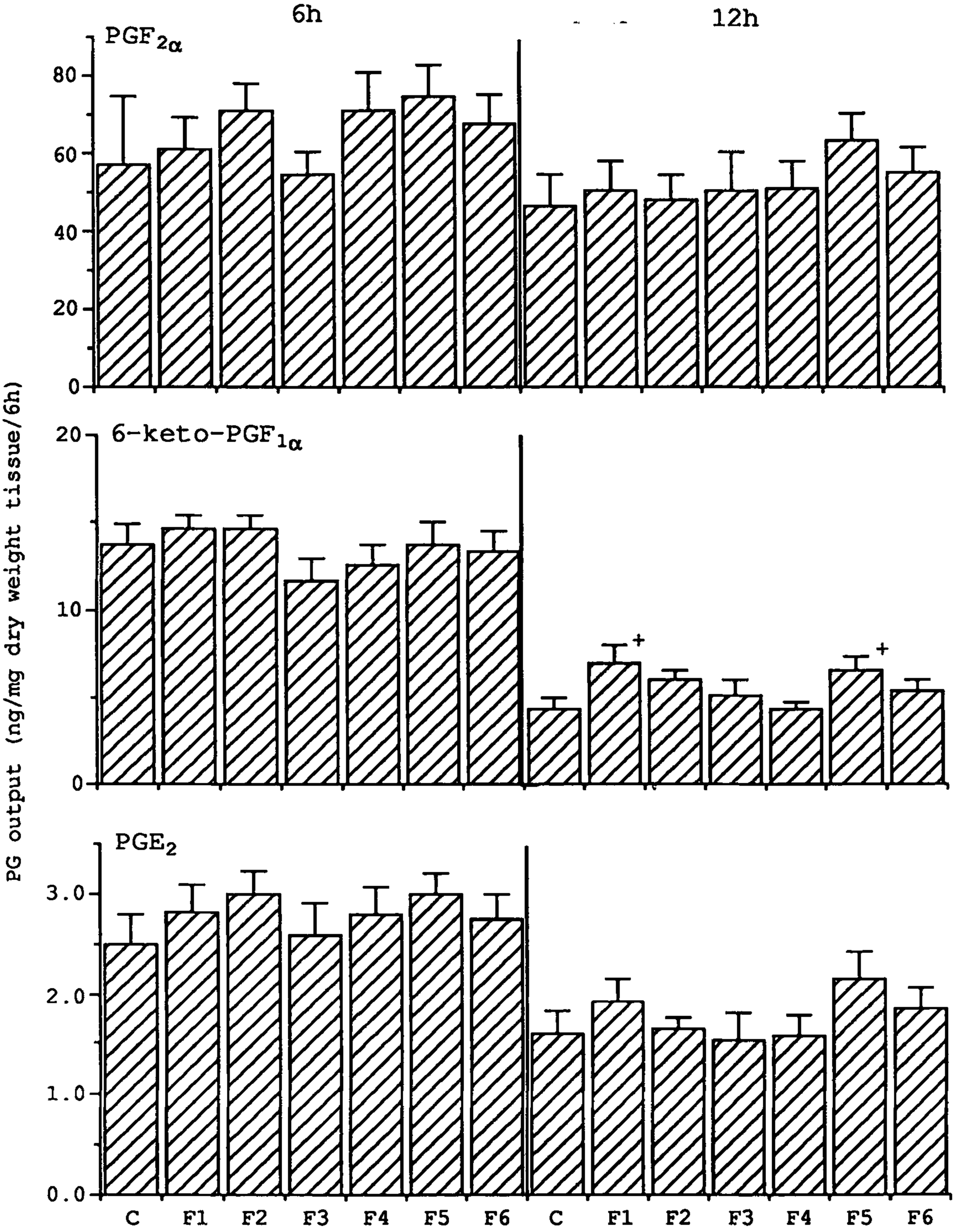


Fig. 48. The effects of F1, F2, F3, F4, F5 and F6 (25µg/ml) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from the Day-15 guinea-pig endometrium cultured for 12h and sampled at 6h. +Significantly ($P < 0.05$) higher than the corresponding control (C) value for the same PG at the same time.

activity of PLA_2.

3:6:e The effects of guinea-pig conceptus secretory proteins on the activity of PLA_2

Introduction

The activity of PLA_2 in the guinea-pig endometrium is the rate-limiting step in endometrial PG synthesis. Therefore, the antiluteolytic factor produced by the guinea-pig conceptus may reduce endometrial $PGF_{2\alpha}$ synthesis by inhibiting the activity of PLA_2. The effects of the protein fractions F1-F6 and unpurified conceptus secretory protein (uCSP) on the activity of cobra venom PLA_2 in an *in vitro* assay were investigated.

Methods

The PLA_2 assay was carried out using phosphatidylcholine (PC) with [^{14}C]-arachidonic acid (AA) in the 2-position as the labelled substrate. 1mg of each of F1-F6 was dissolved in 1ml of PLA_2 assay buffer, containing 0.5U/ml PLA_2 from *Naja naja* venom. This concentration of PLA_2 gives a conversion of [^{14}C]-PC to [^{14}C]-AA of approximately 65% during a 10 min incubation period (Figure 5) and allows for detection of either inhibition or stimulation of PLA_2 activity. 300μl of each protein solution were dispensed in triplicate into Eppendorf tubes. 3 controls were also prepared containing 300μl of PLA_2 assay buffer (0.5U/ml PLA_2) with no protein. The reaction was carried out as described in Section 2:7:(i). The unmetabolised substrate and the fatty acid were separated on silica gel columns using two different solvent systems. [^{14}C]-AA was eluted in hexane/1,4-dioxan/glacial acetic acid (90:10:1) and [^{14}C]-PC was eluted in the more polar solvent, chloroform/methanol/water (65:35:4). The radioactivity in each

solvent fraction was measured by counting in a liquid scintillation counter for 4 min. The conversion of [^{14}C]-PC to [^{14}C]-AA over a 10 min period, calculated as a percentage of the total radioactivity recovered, was used as a measure of PLA_2 activity. The assay was repeated using 1mg/ml uCSP.

Statistical tests

The differences between treated and control groups were analysed by Student's t test or, if the variances of the two groups were significantly different by the variance ratio F test, by a modified t test for unequal variances.

Results

The conversion of [^{14}C]-PC to [^{14}C]-AA by snake venom PLA_2 was unaffected by the presence of F1-F6 (1mg/ml) (Table 11).

The assay was repeated using 1mg/ml of unpurified conceptus secretory protein (uCSP). The conversion of [^{14}C]-PC to [^{14}C]-AA by snake venom PLA_2 in the presence of uCSP (1mg/ml) was 65.29 $\pm$ 4.91%. This was not significantly different from the control conversion of 66.07 $\pm$ 1.87%.

Conclusions

Neither unpurified secretory protein (uCSP) from Day-15 guinea-pig conceptuses nor fractions of protein obtained after separation of PBS by ion-exchange chromatography (F1-F6) had any effect on the activity of snake venom PLA_2 activity in an *in vitro* assay. To further examine the composition of the proteins secreted by the guinea-pig conceptus, F3-F6 were purified into fractions of different molecular weights by gel filtration chromatography.

Table 11. The effects of the Day-15 guinea-pig conceptus secretory proteins F1-F6 on the mean ± s.e.m. ($\underline{n}$ = 3) % conversion of [^{14}C]-PC to [^{14}C]-AA by cobra venom PLA_2.

Protein	% conversion of [^{14}C]-PC to [^{14}C]-AA
Control	46.07 ± 2.37
F1	47.49 ± 3.59
F2	44.41 ± 4.41
F3	52.59 ± 10.73
F4	57.18 ± 4.46
F5	55.85 ± 6.80
F6	52.63 ± 1.67

3:6:f Purification of guinea-pig conceptus secretory proteins by gel filtration chromatography

Introduction

The fractions of guinea-pig conceptus secretory protein purified by ion-exchange chromatography (F1-F6) each contained proteins over a range of molecular weights. It was therefore decided to further purify each fraction by gel filtration chromatography on Sephadex G-75 Superfine (SF). Gel filtration chromatography separates proteins on the basis of molecular size with proteins being eluted from the column in descending order of molecular weight. Small proteins are retarded in their progress through the gel by the presence of pores in the gel beads which they enter. Larger proteins are excluded from the gel pores by size and pass more quickly through the gel. Sephadex G-75 SF has a pore size which results in a fractionation range of 3,000-70,000.

Fractions F3-F6 were run on Sephadex G-75 SF in order to separate high molecular weight proteins (> 70,000) which elute in the void volume, and albumin (67.0kDa) from any low molecular weight proteins present.

Methods

Only fractions F3-F6 were run on Sephadex G-75 SF, as insufficient of fractions F1 and F2 remained after analysis by SDS PAGE and examination of their effects on PG output by Day-15 guinea-pig endometrium and PLA_2 activity.

Each protein was dissolved in 2ml of Sample Buffer and applied to the Sephadex G-75 SF column as described in Section 2:10:(iii). The proteins were collected in 2ml volumes on a Fraction Collector and

separated into fractions on the basis of the peaks of absorbance observed on the chart recording of the elution profile. Each fraction was dialysed, desalted and lyophylized (see Section 2:10) before being weighed. The molecular weights of the proteins in each fraction were determined by SDS PAGE (see Section 2:8).

Results

The elution profiles of fractions F1-F6 from the Sephadex G-75 SF column are shown in Figure 49. F3 exhibited peaks at 80kDa (1) and 66kDa (2) with smaller peaks at 52kDa (3), 35kDa (4) and 16kDa (5). F4 exhibited peaks at 80kDa (1), 66kDa (2) and 35kDa (3). F5 was separated into two fraction with peaks at 80kDa (1) and 70kDa (2). F6 eluted as one peak at 80kDa.

The amount of protein found in each of the fractions into which F3-F6 was separated by gel filtration chromatography on Sephadex G-75 SF is shown in Table 12.

The molecular weight profiles on SDS PAGE of F3-F6 after separation on Sephadex G-75 SF are shown in Figures 50-52. F3:1 contained predominantly high molecular weight proteins with bands at 97.4kDa, 96.5kDa and lightly stained proteins at 74.6kDa and 68.3kDa (Figure 50). F3:2 contained high molecular weight proteins at 96.5kDa and 94.6kDa with large, heavily stained bands at 74.6kDa and 68.3kDa. Staining continued down to 29.6kDa with low molecular weight bands at 15.1kDa and 13.2kDa (Figure 50). F3:3 exhibited protein bands at 74.6kDa and 68.3kDa with further staining down to a diffuse band at 29.6kDa. A heavily stained low molecular weight protein was seen at 15.1kDa (Figure 50). F3:4 contained no high molecular weight proteins and the first bands stained were at 38.2kDa and 30.1kDa with the 15.1kDa protein also being present in

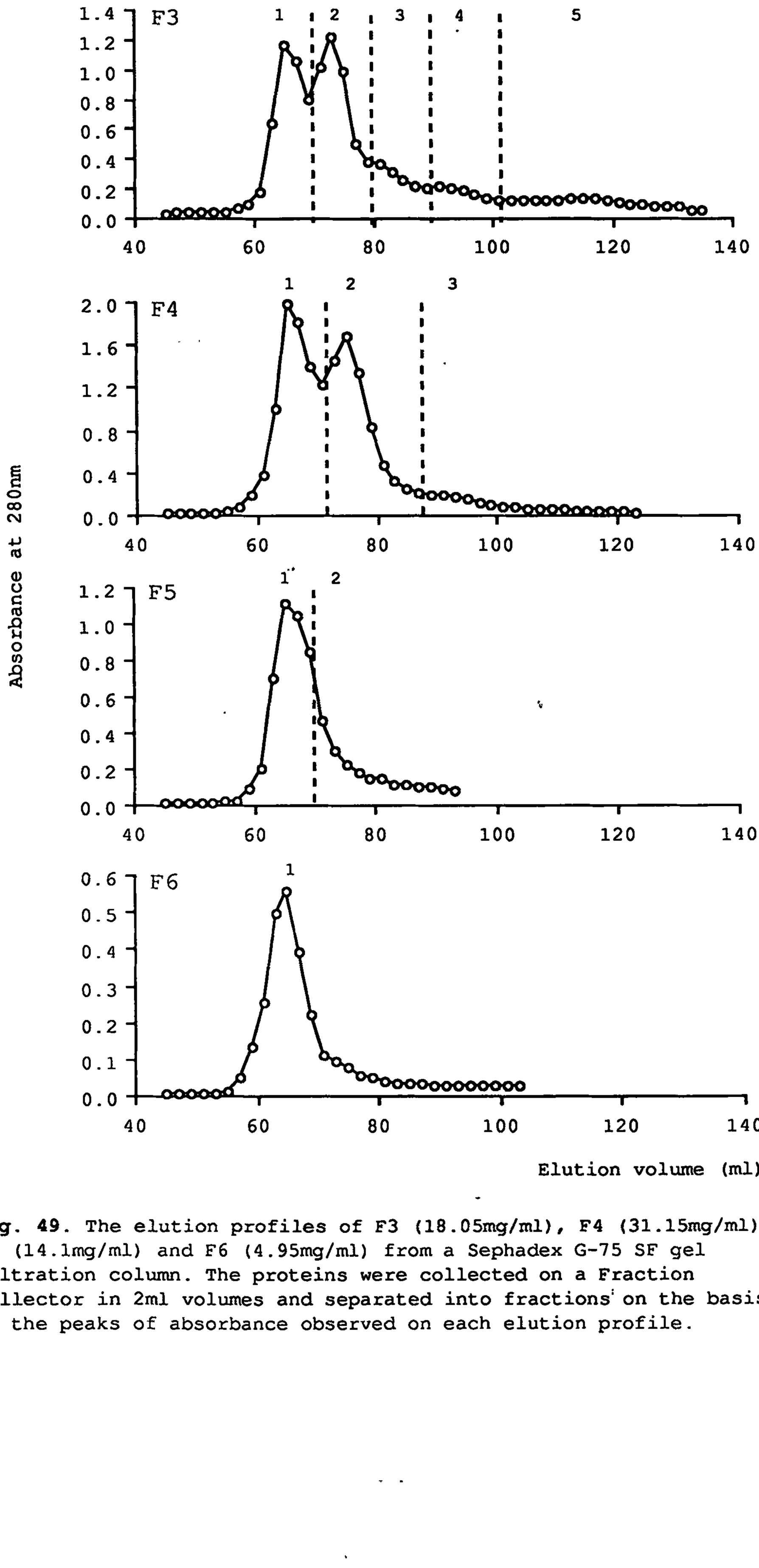


Fig. 49. The elution profiles of F3 (18.05mg/ml), F4 (31.15mg/ml), F5 (14.1mg/ml) and F6 (4.95mg/ml) from a Sephadex G-75 SF gel filtration column. The proteins were collected on a Fraction Collector in 2ml volumes and separated into fractions on the basis of the peaks of absorbance observed on each elution profile.

Table 12. The amounts of protein recovered in the Day-15 guinea-pig conceptus secretory protein fractions F3 (1-5), F4 (1-3), F5 (1-2) and F6 after separation on Sephadex G-75 SF.

	Amount of protein (mg) in each fraction after separation on Sepahadex G-75 SF				
Fraction from ion-exchange	1	2	3	4	5
F3	4.4	9.6	2.0	1.1	1.5
F4	11.9	23.5	3.3		
F5	4.4	7.3			
F6	2.6				

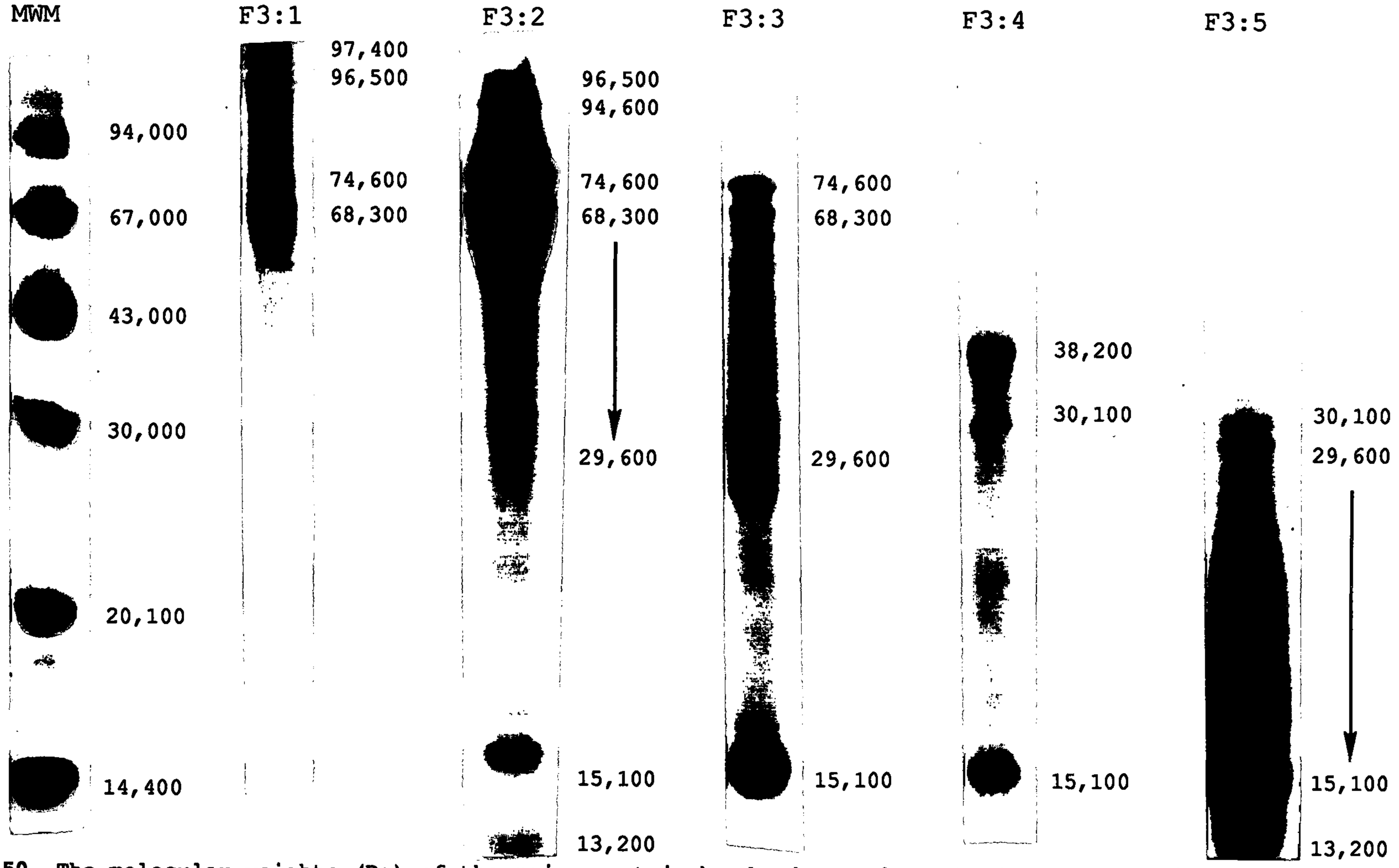


Fig. 50. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig conceptus secretory proteins purified from the PBS fraction, F3:1, F3:2, F3:3, F3:4 and F3:5. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

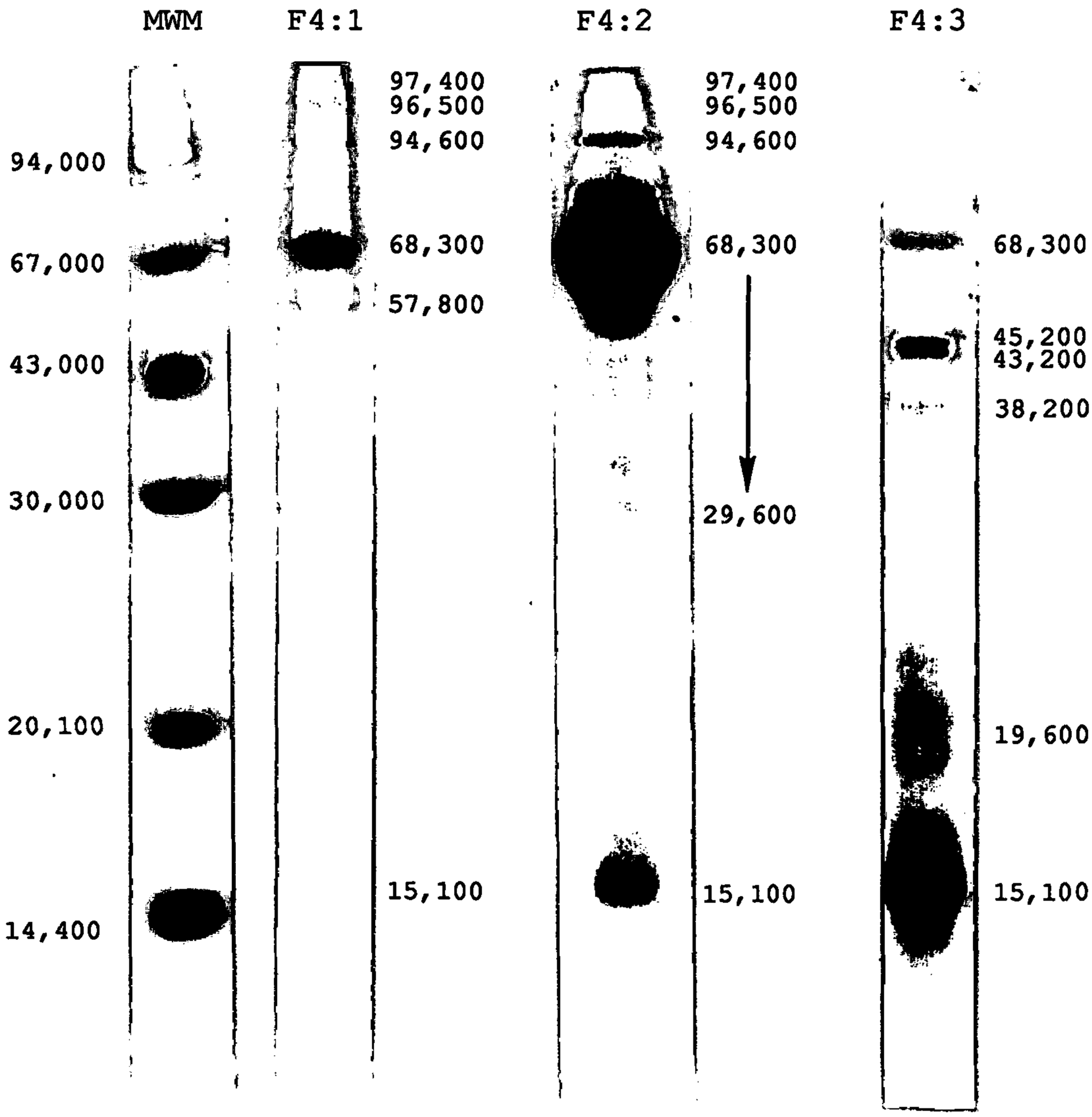


Fig. 51. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig conceptus secretory proteins purified from the PBS fraction, F4:1, F4:2 and F4:3. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

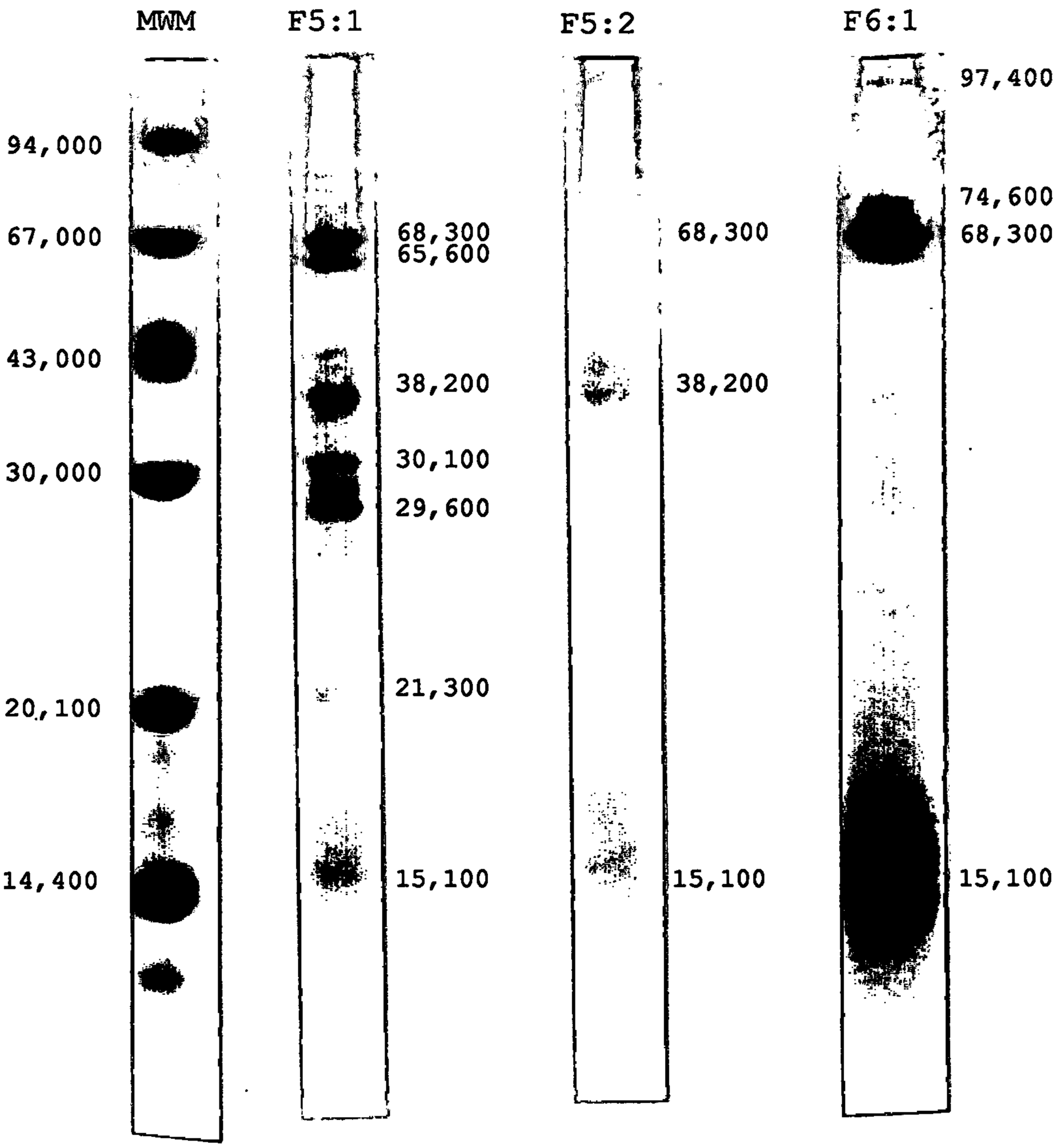


Fig. 52. The molecular weights (Da) of the major protein bands observed on the SDS PAGE profiles of Day-15 guinea-pig conceptus secretory proteins purified from the PBS fraction, F5:1, F5:2 and F6:1. The SDS PAGE profile of molecular weight markers (MWM) run on the same gel is shown for comparison. 10µg of each protein fraction were run on the gel.

this fraction (Figure 50). F3:5-contained lightly stained proteins at 30.1kDa and 29.6kDa with an increase in staining from 28kDa down to an intense diffuse band at 15.1kDa and further staining to 13.2kDa (Figure 50). F4:1 contained high molecular weight proteins at 97.4kDa, 96.5kDa and 94.6kDa with further bands at 68.3kDa and 57.8kDa. A faint band was also seen at 15.1kDa (Figure 51). F4:2 had proteins at 97.4kDa, 96.5kDa and 94.6kDa. There was also a very heavily stained, diffuse band at 68.3kDa with staining continuing down to 29.6kDa. A low molecular weight protein was present at 15.1kDa (Figure 51). F4:3 had a band at 68.3kDa and intermediate molecular weight proteins at 45.5kDa, 43.2kDa and 38.2kDa. Staining increased from the 38.2kDa band down to heavily stained diffuse bands at 19.6kDa and 15.1kDa (Figure 51). F5:1 contained bands at 68.3kDa and 65.6kDa. Intermediate molecular weight proteins were found at 38.2kDa, 30.1kDa and 29.6kDa with lightly stained bands at 21.3kDa and 15.1kDa (Figure 52). F5:2 contained only lightly stained bands at 68.3kDa, 38.2kDa and 15.1kDa (Figure 52). F6:1 contained proteins at 97.4kDa, 74.6kDa, a heavily stained band at 68.3kDa and a diffuse heavily stained band at 15.1kDa (Figure 52).

Conclusions

Gel filtration chromatography on Sephadex G-75 SF successfully resulted in the resolution of F3-F5 into different fractions on the basis of molecular weight as demonstrated by the molecular weight profiles obtained by SDS PAGE analysis of each fraction. The majority of the albumin in F3 and F4 was found in F3:2 and F4:2, respectively, and a large band at approximately the molecular weight of transferrin (76.5kDa) was also seen in F3:2. Heavily stained bands of low molecular weight proteins at 15.1kDa were observed in

F3:3, F3:5, F4:2, F4:3 and F6:1. The identity of these proteins is unknown. Each of the fractions purified by gel filtration chromatography on Sephadex G-75 SF was tested for its effects on the output of PGs from Day-15 non-pregnant guinea-pig endometrium in culture.

3:6:g The effects of uCSP, PBS, ROBS, and fractions of guinea-pig conceptus secretory proteins separated by gel filtration chromatography, on the output of prostaglandins from Day-15 guinea-pig endometrium in culture

Introduction

The protein fractions tested previously for their effects on PG output from Day-15 non-pregnant guinea-pig endometrium were purified from the fraction of guinea-pig conceptus secretory protein which exhibited no affinity for Blue Sepharose (PBS). However it may be that the fraction of guinea-pig conceptus secretory protein which bound to the Blue Sepharose column (ROBS) is responsible for the antiluteolytic effect of the guinea-pig conceptus. It was therefore decided to examine the effects of the ROBS fraction as well as the uCSP and PBS fractions and the fractions of F3-F5 obtained after separation by gel filtration chromatography on the output of PGs by Day-15 guinea-pig endometrium in culture.

Methods

The uteri were removed from five Day-15 guinea-pigs and, under aseptic conditions, each uterine horn was "opened" by a longitudinal incision. The endometrium was dissected away from the myometrium and cut into 1-2mm^3 pieces. 28 petri dishes were prepared containing 5-10mg wet weight (1-2mg dry weight) of endometrium from each uterus. Pairs of dishes were treated with uCSP (500μg/ml), PBS (450μg/ml), ROBS (350μg/ml), F3:1 (100μg/ml), F3:2 (200μg/ml), F3:3 (50μg/ml), F3:4 (25μg/ml), F3:5 (35μg/ml), F4:1 (200μg/ml), F4:2 (400μg/ml), F4:3 (50μg/ml), F5:1 (100μg/ml), F5:2 (150μg/ml) and no protein (control). Each protein treatment was used at the maximum

concentration possible consistent with the amount of protein available for the procedure being sufficient for the cultures to be carried out in duplicate, over two time periods on five animals. In addition, to allow exposure of the tissue to as high a concentration of each protein as possible, the volume of culture medium in each petri dish was reduced from 4ml to 2ml by the use of 3.5cm diameter dishes. The petri dishes were incubated at 37°C for 12h and the culture medium was removed and replaced with fresh medium containing the same protein treatments at 6h. The samples of culture medium obtained were stored at -20°C before being assayed for $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 by radioimmunoassay (see Section 2:9). After culture, the pieces of endometrium were removed from each petri dish into separate preweighed containers and dried by placing in an oven at 37°C for 24h. Each container was reweighed and the amount of dried endometrium from each dish was calculated. The outputs of PGs were calculated per mg dry weight of tissue.

Statistical tests

Changes in the output of PGs with time and differences between control and treated groups were analysed by Student's *t* test or, if the variances of the two groups were significantly different by the variance ratio *F* test, by a modified *t* test for unequal variances.

Results

The control outputs of 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 endometrium significantly ($P < 0.05$) declined during 12h of culture (Figure 53). The control output of $PGF_{2\alpha}$ from Day-15 endometrium was unchanged during 12h of culture (Figure 53).

The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15

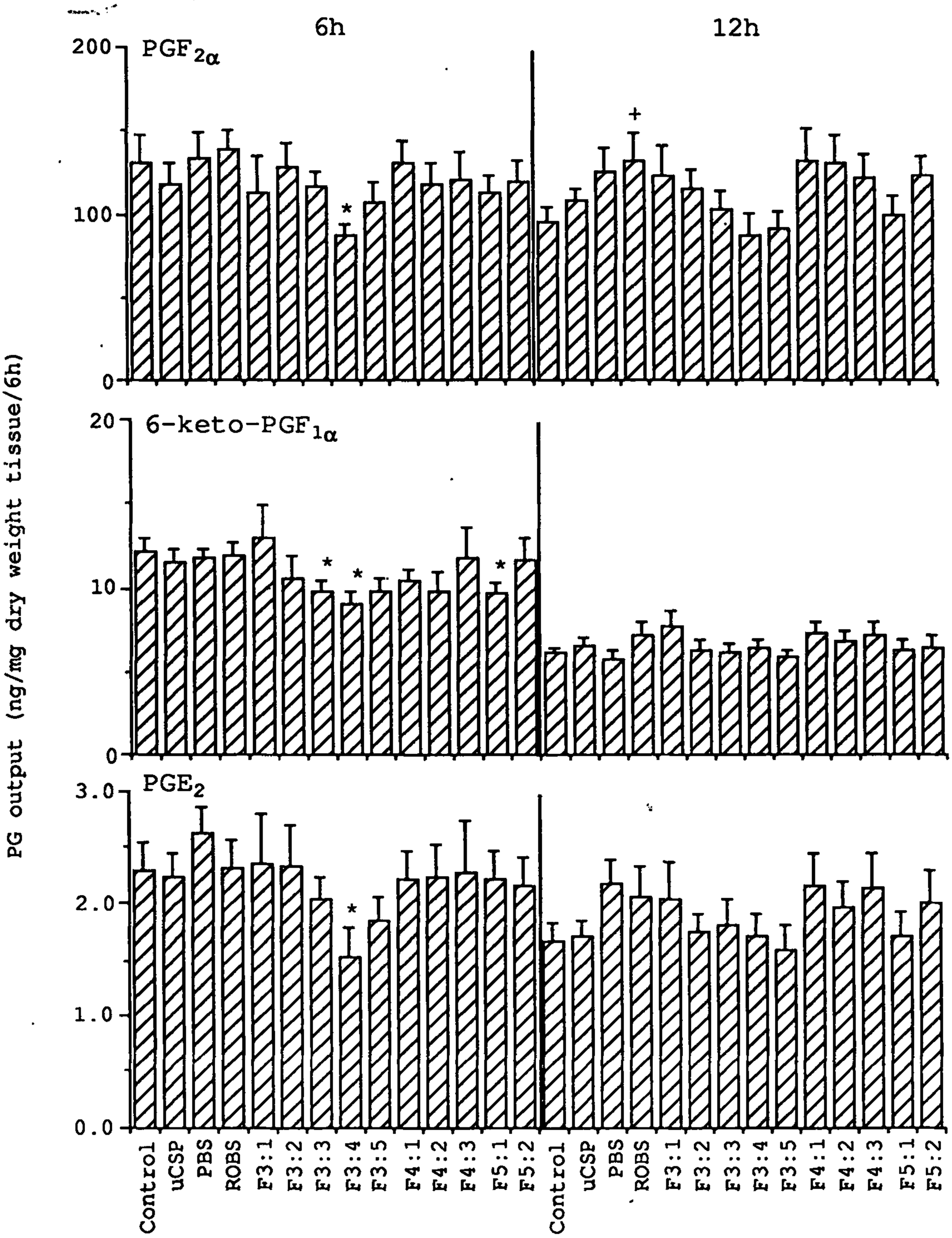


Fig. 53. The effects of uCSP (500µg/ml), PBS (450µg/ml), ROBS (350µg/ml), F3:1 (100µg/ml), F3:2 (200µg/ml), F3:3 (50µg/ml), F3:4 (25µg/ml), F3:5 (35µg/ml), F4:1 (200µg/ml), F4:2 (400µg/ml), F4:3 (50µg/ml), F5:1 (100µg/ml) and F5:2 (150µg/ml) on mean (± s.e.m., n = 10) outputs of prostaglandin (PG) $F_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-15 guinea-pig endometrium cultured for 12h and sampled at 6h. *Significantly ($P < 0.05$) lower than the corresponding control value for the same PG at the same time.+Significantly ($P < 0.05$) higher than the corresponding control value for the same PG at the same time.

guinea-pig endometrium were significantly ($P < 0.05$) inhibited by F3:4 (25μg/ml) (Figure 53). In addition, the output of 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium was significantly ($P < 0.05$) inhibited by F3:3 (50μg/ml) and F5:1 (100μg/ml) at 6h (Figure 53). None of the protein fractions affected the output of PGs from Day-15 endometrium at 12h, except ROBS which significantly stimulated ($P < 0.05$) the output of $PGF_{2\alpha}$ from Day-15 endometrium at 12h (Figure 53).

Conclusions

F3:4 (25μg/ml) inhibited the outputs of all 3 PGs from Day-15 endometrium at 6h of culture. This was the lowest concentration of any protein fraction tested which gives added significance to the result. However, the inhibition of PG synthesis by F3:4 was not continued to 12h of culture. Neither was the inhibition of 6-keto-$PGF_{1\alpha}$ output from Day-15 endometrium at 6h by F3:3 and F5:1 continued to 12h. Therefore, it seems unlikely that these proteins are responsible for the sustained depression in endometrial $PGF_{2\alpha}$ synthesis in the pregnant guinea-pig. These results do provide further evidence that endometrial PGI_2 synthesis is controlled differently from endometrial $PGF_{2\alpha}$ and PGE_2 synthesis as neither $PGF_{2\alpha}$ nor PGE_2 output from Day-15 guinea-pig endometrium was affected by F3:3 and F5:1.

DISCUSSION

Proteins purified from the culture medium from the Day-15 guinea-pig conceptus were found to contain large amounts of serum albumin. Similar serum protein contamination was found in protein isolated from culture medium from Day-15 non-pregnant guinea-pig

endometrium (see Section 3:3). It therefore appears that serum proteins leach into the uterine lumen of the guinea-pig and become associated with the endometrium and conceptus.

None of the protein fractions purified from the guinea-pig conceptus by ion-exchange chromatography were capable of reducing the output of PGs from Day-15 guinea-pig endometrium in culture. Neither did these protein fractions or unpurified conceptus secretory protein affect the activity of cobra venom PLA_2. However after further purification by gel filtration chromatography, F3:4 was found to inhibit the output of all three PGs, and F3:3 and F5:1 inhibited the output of 6-keto-$PGF_{1\alpha}$ from Day-15 guinea-pig endometrium in culture at 6h. F3:4 contained proteins in the molecular weight range 38.2-15.1kDa with a more heavily stained band at 38.2kDa than observed on the SDS PAGE profile of any of the other protein fractions. It appears, therefore, that this 38.2kDa protein may be responsible for the inhibition of PG synthesis by F3:4. However, none of the effects of the protein fractions which did inhibit PG synthesis from Day-15 endometrium was large enough or continued for long enough to account for the reduction in endometrial $PGF_{2\alpha}$ synthesis in pregnant guinea-pigs compared to cyclic guinea-pigs on Day-15. It may be that the endometrium requires to be exposed to conceptus secretory proteins for longer than 12h and at higher concentrations than used in this experiment in order for endometrial PG synthesis to be affected. However, the amounts of protein isolated from the guinea-pig conceptus were too small to allow longer cultures to be carried out.

The stimulation of 6-keto-$PGF_{1\alpha}$ output by F1 and F5 and of $PGF_{2\alpha}$ by ROBS from Day-15 endometrium in culture may have been due to these proteins binding to fatty acids and relieving product-

inhibition of PLA_2 (Conricode and Ochs, 1989). However, the stimulation of 6-keto-$PGF_{1\alpha}$ output from the Day-15 endometrium by F1 and F5 was not reflected by an increase in the activity of PLA_2 from cobra venom in an in vitro assay. These results suggest that either F1 and F5 did not stimulate endometrial 6-keto-$PGF_{1\alpha}$ output by an action on endometrial PLA_2 or that the activity of PLA_2 in the guinea-pig endometrium may be regulated differently from the activity of snake venom PLA_2 in vitro. The exact composition of the intracellular environment to which endometrial PLA_2 is exposed is unknown and may modify the effects of any PLA_2 stimulatory or inhibitory factors compared to their effects in vitro.

SECTION 4

GENERAL DISCUSSION

The output of $PGF_{2\alpha}$ from Day-7 endometrium and the outputs of $PGF_{2\alpha}$ and PGE_2 from Day-15 endometrium were inhibited by sodium fluoride (10mM) during a 24h period of tissue culture. The output of 6-keto-$PGF_{1\alpha}$ from Day-15 endometrium was also inhibited by sodium fluoride when sodium fluoride treatment was repeated at 6-hourly intervals rather than at 8-hourly intervals over 24h of culture. Sodium fluoride (10mM) also caused an inhibition of [^{3}H]-leucine incorporation into cellular and secreted proteins in Day-15 guinea-pig endometrium in culture for 24h. Thus as far as endometrial protein and prostaglandin synthesis are concerned, sodium fluoride caused similar effects to the protein synthesis inhibitors actinomycin D, cycloheximide and puromycin (Riley and Poyser, 1989). Sodium fluoride inhibits protein synthesis by causing dissociation of polyribosomes to 80S ribosomes and ribosomal subunits with the loss of nascent chains from ribosomes (Marks, Burka, Conconi, Perl and Rifkind, 1965). During this polyribosome breakdown, there is conservation of mRNA as indicated by the restoration of protein synthesis upon removal of sodium fluoride and reincubation in the presence of glucose. Inhibition of protein synthesis and polyribosomal decay may be secondary to a block in the generation of high energy phosphate compounds, such as ATP, as sodium fluoride blocks glucose metabolism by inhibiting the enzyme enolase (Lohmann and Meyerhof, 1934) which is responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis. Enolase requires magnesium as a co-factor and inhibition of enolase by fluoride is due to the formation of an inactive magnesium-fluoro-phosphoenolase complex (Najjar, 1948). However,

sodium fluoride inhibition of protein synthesis is not due to magnesium removal (Colombo, Vesco and Baglioni, 1968). Therefore, the inhibition of protein synthesis caused by sodium fluoride in the guinea-pig endometrium is probably due to a direct effect of sodium fluoride on 80S ribosomes rather than the general consequence of a breakdown in cellular metabolism due to sodium fluoride-induced inhibition of ATP formation.

The particularly strong inhibition of $PGF_{2\alpha}$ output from guinea-pig endometrium by sodium fluoride provides further evidence that the synthesis of $PGF_{2\alpha}$ is controlled by a protein which is stimulated by oestradiol acting on a progesterone-primed uterus. However sodium fluoride had no effect on 6-keto-$PGF_{1\alpha}$ output from Day-15 endometrium until the treatment was repeated a third time during 18h of culture suggesting that the synthesis of PGI_2 is less dependent on fresh protein synthesis than either $PGF_{2\alpha}$ or PGE_2 synthesis. This agrees with the findings of Riley and Poyser (1989) who showed that 6-keto-$PGF_{1\alpha}$ output from the guinea-pig endometrium in culture was relatively unaffected by the protein synthesis inhibitors actinomycin D, cycloheximide and puromycin. In addition progesterone, which was also shown to have inhibitory effects on endometrial $PGF_{2\alpha}$ and protein synthesis in culture, had no effect on the endometrial output of 6-keto-$PGF_{1\alpha}$ (Riley and Poyser, 1990). Indeed, it has been demonstrated in this thesis and in other studies (Riley and Poyser, 1989, 1990) that the output of 6-keto-$PGF_{1\alpha}$ from guinea-pig endometrium in culture remains virtually unchanged between Day 7 and Day 15 of the cycle, and therefore the protein which is proposed to stimulate $PGF_{2\alpha}$ synthesis at the end of the cycle has no effect on endometrial PGI_2 synthesis.

In the short term the effect of sodium fluoride on endometrial prostaglandin output is stimulatory. The outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 from Day-7 guinea-pig endometrium in culture for 1h and from Day-7 and Day-15 guinea-pig uterus superfused in vitro for 30 min were increased by sodium fluoride (10mM) treatment. 6-Keto-$PGF_{1\alpha}$ output was also increased from Day-7 endometrium at 16h of culture and from Day-15 endometrium at 6h and 8h of culture. These results implied that a G-protein might be involved in regulating uterine prostaglandin output in the guinea-pig. However, the G-protein modulators pertussis toxin and cholera toxin had no effect on the output of prostaglandins from either the Day-7 or the Day-15 guinea-pig endometrium in culture for 24h. The stimulation of prostaglandin output from the superfused Day-7 uterus by sodium fluoride is characterised by a lag period of 10-20 min and has been shown to be due probably to the release of calcium from intracellular stores since the effect of sodium fluoride was blocked by TMB-8 (an intracellular calcium antagonist). The sodium fluoride-stimulated prostaglandin output from the superfused Day-7 uterus is unaffected by either lack of extracellular calcium or the calmodulin inhibitors trifluoperazine and W-7. The release of intracellular calcium caused by sodium fluoride in human neutrophils (Strnad and Wong, 1985) and rat pancreatic acini (Matozaki, Sakamoto, Nagao, Nishizaki and Baba, 1988) is characterized by a similar lag period and insensitivity to pertussis toxin and cholera toxin, as is the sodium fluoride-mediated stimulation of prostaglandin synthesis in the guinea-pig uterus. Similarly, the sodium fluoride-stimulated generation of leukotrienes and 12-HETE from platelets exhibits a lag period and is not influenced by cholera or pertussis toxin (Brom,

Koller, Brom and Konig, 1989), and the sodium fluoride-stimulated PGI_2 synthesis in cultured human umbilical vein is neither dependent on extracellular calcium (Garcia, Painter, Fenton, English and Callahan, 1990) nor is it affected by pertussis toxin (Magnusson, Halldorsson, Kjeld and Thorgeirsson, 1989). The stimulation of LH release by sodium fluoride, a Ca^{2+} dependent mechanism, from primary pituitary cell cultures from immature female rats is also insensitive to pertussis and cholera toxin (Waters, Hawes and Conn, 1990). Therefore, in several tissues the sodium fluoride-mediated release of intracellular calcium or increase in the synthesis of metabolites of arachidonic acid is mediated by a toxin-insensitive mechanism and exhibits a lag period similar to that seen during the sodium fluoride-stimulated increase in prostaglandin synthesis in the guinea-pig uterus. Consequently, stimulation of prostaglandin output from the guinea-pig uterus by sodium fluoride may be due to activation of a G-protein which controls the release of calcium from intracellular stores. The sodium fluoride-induced stimulation of $PGF_{2\alpha}$ output from the superfused Day-7 uterus exhibits a similar independence of extracellular calcium and calmodulin as the high basal output of $PGF_{2\alpha}$ from the superfused Day-15 uterus (Poyser, 1984b, 1985a, 1985b). Therefore sodium fluoride may mimic the mechanism by which $PGF_{2\alpha}$ output is stimulated in the Day-15 uterus <u>in vivo</u> by the presence of oestradiol acting on a progesterone-primed uterus. Consequently, oestrogen acting on a progesterone-primed uterus may induce the formation of a G-protein, or of a protein which activates a G-protein, which controls the release of intracellular calcium. G-protein expression has been shown to be regulated by thyroid hormones (Rapiejko, Watkins, Ros and Malbon, 1989; Milligan and

Saggerson, 1990; Levine, Feldman, Robishaw, Ladenson, Ahn, Moroney and Smallwood, 1990; Saunier, Dib, Delemer, Jacquemin and Correze, 1990) and glucocorticoids (Saito, Guitart, Hayward, Tallman, Duman and Nestler, 1989; Haigh, Jones and Milligan, 1990). As oestrogen and progesterone receptors belong to the same superfamily of nuclear receptors as glucocorticoid and thyroid hormone receptors it is possible that these steroid hormones can also control G-protein expression.

Since PGH synthase occurs largely in the endoplasmic reticulum (DeWitt, Rollins, Day, Gauger and Smith, 1981) and PLA_2 is often associated with intracellular membranes (Lagarde, Menashi and Crawford, 1981; Kurihara, Nakano, Takasu and Arita, 1991), and since there is much calcium bound in the endoplasmic reticulum, it is possible that sodium fluoride acts directly on the endoplasmic reticulum to mobilise calcium and thereby stimulate prostaglandin synthesis. This may be due to the sodium fluoride-mediated activation of a G-protein, as (i) a G-protein has been found in the endoplasmic reticulum of the dog pancreas (Audigier, Nigam and Blobel, 1988), (ii) IP_3-mediated Ca^{2+} release from liver microsomes is stimulated by GTP (Dawson, 1985), and (iii) a guanine nucleotide regulatory mechanism may mediate release of Ca^{2+} from the endoplasmic reticulum of the neuronal cell line NIE-115 (Gill, Ueda, Chueh and Noel, 1986).

Many receptor-mediated effects involve the release of calcium from intracellular stores. Agonist-induced rises in intracellular free calcium have been associated with an enhancement of polyphosphoinositide hydrolysis (Michell, 1975), with the resultant rapid generation of water soluble inositol phosphates. Inositol 1,4,5-trisphosphate (IP_3) was subsequently shown to release

calcium from non-mitochondrial stores in permeabilized pancreatic acini (Streb et al., 1983). The intracellular calcium-mobilizing action of IP_3 was confirmed in many other cell types including hepatocytes (Burgess, Godfrey, McKinney, Berridge, Irvine and Putney, 1984; Joseph, Thomas, Williams, Irvine and Williamson, 1984), neutrophils (Prentki, Wollheim and Lew, 1984), Swiss 3T3 cells (Berridge, Heslop, Irvine and Brown, 1984) and insulin-secreting cells (Biden, Prentki, Irvine, Berridge and Wollheim, 1984). IP_3 binds to a receptor on the endoplasmic reticulum (Spat, Bradford, McKinney, Rubin and Putney, 1986) and opens a calcium channel in the endoplasmic reticulum membrane (Muallem, Schoffield, Pandol and Sachs, 1985). A G-protein has been implicated in the receptor-mediated breakdown of PIP_2 by PLC (Gomperts, 1983: Haga, Haga, Ichiyama, Katada, Kurose and Ui, 1985; Nakamura and Ui, 1985; Haslam and Davidson, 1984; Litosch, Wallis and Fain, 1985). Thus the fluoride-mediated stimulation of prostaglandin formation in the guinea-pig uterus, which these studies have shown to be dependent on the release of calcium from intracellular stores, could be due to activation of a G-protein linked to PLC with the subsequent formation of IP_3 and the release of intracellular calcium. However, experiments in this thesis have shown that neomycin, a PLC inhibitor, has no effect on the fluoride-mediated stimulation of prostaglandin release from the guinea-pig uterus suggesting that fluoride is not acting by stimulating a G-protein linked to PLC in this case.

Guanine nucleotides have been shown to release intracellular calcium by a different mechanism than that by which IP_3 mediates calcium release. Guanosine triphosphate (GTP) and IP_3 both stimulate calcium release from rat liver microsomes and guinea-pig

parotid gland microsomal subfractions (Henne and Soling, 1986). GTP-mediated calcium release is blocked by non-hydrolysable analogues of GTP, implicating the presence of a G-protein, whereas IP_3-mediated calcium release is unaffected by these compounds. In addition, (i) GTP-induced release of calcium is dependent upon the presence of compounds which increase the viscosity of the medium (i.e. polyethylene glycol, polyvinylpyrrolidone and bovine serum albumin), (ii) GTP-induced release of calcium is much slower than IP_3-mediated calcium release, (iii) GTP-mediated but not IP_3-mediated calcium release is strongly temperature dependent, and (iv) GTP-mediated calcium release is much more sensitive to a decrease in intravesicular free calcium concentrations than IP_3-mediated calcium release. Similar distinctions between GTP- and IP_3-induced release of intracellular calcium have been found in saponin-permeabilized NIE-115 cells (Chueh and Gill, 1986) and subcellular fractions of neuroblastoma x glioma (NG108-15) hybrid cells (Jean and Klee, 1986). In addition, GTP-sensitive and IP_3-sensitive calcium pools have been shown to reside in different intracellular compartments (Henne, Piiper and Soling, 1987). The IP_3-sensitive pool was recovered together with the plasma membrane fraction and the GTP-sensitive pool was located in a subfraction of the rough endoplasmic reticulum. Thus, fluoride-mediated stimulation of prostaglandin output from the guinea-pig uterus may be due to the activation of a G-protein on the rough endoplasmic reticulum resulting in the release of intracellular calcium and the activation of PLA_2. This would fit in with the lag period seen in the sodium fluoride-stimulated release of prostaglandins from the guinea-pig uterus and the presence of PGH synthase on the endoplasmic reticulum.

Other proteins besides G-proteins have been discovered which stimulate PLA_2. Melittin, the principal component of the venom of the common honey bee (Habermann, 1972), increases the rate of bee venom PLA_2-catalyzed hydrolysis of sonicated liposomes of egg phosphatidylcholine (Mollay and Kreil, 1974). The structural organisation of the melittin molecule (Mr 2,800 Da) is unusual in that there is significant segregation of polar and nonpolar amino acids and there are no anionic sites. Consequently, melittin has the structural organisation of a surfactant and is strongly surface active and membrane lytic. Melittin probably stimulates PLA_2 by interacting with organised aggregates of phosphatidylcholine in such a way as to make these aggregates more suitable substrates for enzymatic attack (Yunes et al., 1977). Similar PLA_2-stimulatory proteins have been found in venom from various species and these include the bacterial toxin polymyxin B (Mollay and Kreil, 1974), direct lytic factor from cobra venom (Shier, 1979), mastorapan from wasp venom (Argiolas and Pisano, 1983) and venom from the acontial nematocysts of the sea anemone (Hessinger and Lenhoff, 1976).

Antibodies against melittin have been used to identify and isolate a protein with similar functional and antigenic properties in mammalian cells (Clark, Conway, Shorr and Crooke, 1987). The PLA_2-activating protein (PLAP; Mr 28,000 Da) was found in the murine smooth muscle cell line BC3Hi, the bovine endothelial cell line CPAE, the human monocytic cell line U937 and the murine T-cell line EL4. PLAP was found to stimulate bee venom PLA_2 but had no effect on PLC activity or on the activity of PLA_2 from the pig pancreas or snake venom. In addition, PLAP selectively stimulated PLA_2 when phosphatidylcholine was used as a substrate but had no effect when phosphatidylethanolamine was used as a substrate. Tumour

necrosis factor (TNF), a polypeptide cytokine produced by monocytes and macrophages in response to endotoxin and other immune and inflammatory stimuli, increased PLA_2 activity and [^{3}H]-arachidonic acid metabolite synthesis in the bovine endothelial cell line CPAE with an associated increase in the synthesis of PLAP (Clark, Chen, Crooke and Bomalaski, 1988). Both cycloheximide and actinomycin D inhibited the increase in PLA_2 activity and PLAP formation in response to TNF, as did pertussis toxin also. Thus, TNF-induced activation of PLA_2 requires the synthesis of PLAP and may be mediated by a G-protein. Other stimuli associated with inflammation, such as leukotriene D_4 and interleukin 1, have also been shown to increase the synthesis of PLAP.

PLAP has been isolated from rheumatoid synovial fluid (Bomalaski, Baker, Resurreccion and Clark, 1989a) and gouty synovial fluid (Bomalaski, Baker, Brophy and Clark, 1990a). The stimulation of intracellular and secretory PLA_2 activities by monosodium urate crystals in human peripheral blood neutrophils and monocytes was associated with an increase in PLAP (Bomalaski et al., 1990a). Examination of rheumatoid joint tissue, with the use of immunohistochemical techniques, demonstrated a high concentration of PLAP in monocytes, macrophages, chondrocytes, vascular smooth muscles and endothelial cells (Bomalaski, Fallon, Turner, Crooke, Meunier and Clark, 1990b). In addition, purified PLAP injected into rabbit knee joints resulted in an acute inflammatory arthritis with synovial cell proliferation, synovial fluid leukocytosis and the induction of prostaglandin formation. Thus PLAP is capable of inducing inflammatory states, activating PLA_2 and increasing prostaglandin formation in vivo. An exuberant release of lysosomal

enzymes and superoxide ion have been associated with the pathogenesis of rheumatoid arthritis and PLAP induced human neutrophils to produce superoxide ions and induced neutrophil aggregation also (Bomalaski, Baker, Brophy, Resurreccion, Spilberg, Munian and Clark, 1989b). Consequently, PLAP appears to be involved in the disordered regulation of PLA_2 found in chronic immune and inflammatory states.

A further group of PLA_2-activating proteins which appear to be steroid-controlled have been identified. Incubation of calf thymocytes with testosterone produces an increase in a family of proteins which stimulate the activity of PLA_2 from snake venom and pig pancreas (Goldman, Katsumata and Goto, 1988). The formation of these proteins, known as lipokinins is blocked by cycloheximide. Lipokinins may, however, be distinct from PLAP and melittin as neither PLAP nor melittin activates snake venom PLA_2. Lipokinins appear to mediate testosterone-induced masculine genital differentiation in embryonic mice which is thought to be due to activation of the arachidonic acid cascade. A PLA_2-stimulatory protein (Mr 63,000 Da) with a similar masculinizing effect has been found in the foetal genital tracts of male and testosterone-exposed female mice (Gupta and Braun, 1990). The discovery of steroid-controlled protein activators of PLA_2 supports the hypothesis of oestradiol acting on a progesterone-primed uterus stimulating the formation of a protein which activates PLA_2 in the guinea-pig endometrium.

It appears that there are a number of proteins which are capable of stimulating PLA_2 and which may or may not be related to each other. Whether any of these proteins is involved in the regulation of PLA_2 activity, and consequently prostaglandin formation, in the

guinea-pig endometrium is unknown. However, melittin has been shown to stimulate the outputs of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, but not of PGE_2, from the Day-7 guinea-pig uterus superfused in vitro (Johnson and Poyser, 1991). This effect of melittin was partially reduced by the removal of calcium from the Krebs' solution superfusing the uterus and was completely prevented by the intracellular calcium antagonist TMB-8. Trifluoperazine was without effect on the melittin-induced stimulation of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ outputs from the Day-7 uterus, suggesting that calmodulin is not involved in mediating the effect of melittin. Sublytic concentrations of melittin also activate PLA_2 in mouse 3T3-4a fibroblasts with the resultant release of arachidonic acid from phospholipids and conversion of arachidonic acid to prostaglandins (Shier, 1979). Stimulation of PLA_2 by melittin in mouse 3T3-4a fibroblasts was also dependent upon calcium, since the addition of EDTA to the culture medium prevented the increase in prostaglandin production produced by melittin. These results are compatible with the suggestion that there could be a protein which is functionally similar to melittin in the guinea-pig uterus (i.e. PLAP) which is involved in the stimulation of endometrial $PGF_{2\alpha}$ synthesis at the end of the oestrous cycle.

Experiments in this thesis attempted to isolate a protein from Day-15 guinea-pig endometrium which might be involved in the increase in $PGF_{2\alpha}$ output from the guinea-pig uterus at the end of the cycle. Although total endometrial secretory protein did stimulate prostaglandin output from Day-7 guinea-pig endometrium in culture to similar levels as those found in Day-15 endometrium, it was felt that this result was a nonspecific effect of the large amount of protein used (700μg/ml) resulting in a relief of

product-inhibition of endometrial PLA_2 (Conricode and Ochs, 1989). Several fractions of guinea-pig endometrial protein which were purified by affinity, ion-exchange and gel filtration chromatographic techniques were found to stimulate the activity of cobra venom PLA_2 in an <u>in vitro</u> assay. The proteins present in these fractions, which were not present on the SDS PAGE profiles of other endometrial secretory protein fractions, included proteins at 19.6kDa, 15.6kDa, 14.5kDa, 13.9kDa and 13.2kDa. It therefore seems possible that a low molecular weight (<20.0kDa) protein may be responsible for the stimulation of endometrial $PGF_{2\alpha}$ output which occurs in the guinea-pig at the end of the cycle. Cellular proteins from Day-15 guinea-pig endometrium did not affect the output of prostaglandins from Day-7 guinea-pig endometrium or the activity of cobra venom PLA_2 implying that an intracellular protein is not directly responsible for the increase in endometrial $PGF_{2\alpha}$ output in the guinea-pig at the end of the cycle. Indeed only the incorporation of $[^3H]$-leucine into secreted proteins, not cellular proteins, was increased between Day-7 and Day-15 of the cycle (Riley and Poyser, 1989). It is therefore proposed that oestradiol acting on a progesterone-primed uterus stimulates the synthesis of a low molecular weight secretable protein in the guinea-pig endometrium which activates a G-protein on the rough endoplasmic reticulum resulting in the release of calcium from intracellular stores. Endometrial PLA_2 is then activated by the increased levels of intracellular calcium with the resultant release of arachidonic acid from membrane lipids and the formation of $PGF_{2\alpha}$.

Studies in this thesis have also investigated the mechanism by which the guinea-pig conceptus prevents the increase in uterine $PGF_{2\alpha}$ synthesis which normally occurs at the end of the cycle

resulting in the prevention of luteal regression in the pregnant guinea-pig. It has previously been demonstrated that the synthesis of $PGF_{2\alpha}$ is lower in the uterus of the pregnant guinea-pig than the non-pregnant guinea-pig (Maule Walker and Poyser, 1973, 1974; Blatchley et al., 1975a, 1975b; Antonini et al., 1976; Poyser, 1984a). Experiments in this thesis have shown that the outputs of $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and PGE_2 were 12.1-, 2.1- and 2.6-fold higher respectively from Day-15 cyclic endometrium than from Day-15 pregnant endometrium. Therefore, these results confirm that prostaglandin output and in particular $PGF_{2\alpha}$ output from the guinea-pig endometrium is suppressed during pregnancy.

The activity of PLA_2 is the rate-limiting step in prostaglandin synthesis, and this enzyme is therefore a likely target for the antiluteolytic factor from the guinea-pig conceptus to exert its inhibitory effect on uterine $PGF_{2\alpha}$ synthesis. The activity of PLA_2 in the rat uterus has been shown to be regulated by steroidal hormones. PLA_2 activity in the hypophysectomized or ovariectomized rat uterus is low and remains unaltered by treatment with progesterone (Dey, Hoversland and Johnson, 1982; Pakrasi, Cheng and Dey, 1983). However, treatment with oestradiol results in a large increase in PLA_2 activity. A single oestradiol injection 24h after termination of progesterone treatment increased uterine PLA_2 activity, whereas injection of oestradiol at the same time as the final progesterone treatment was without effect on uterine PLA_2 activity. The PLA_2-stimulating effect of oestradiol was blocked by cycloheximide indicating that oestradiol mediates its effect by inducing fresh protein synthesis. The activity of PLA_2 in the human endometrium varies throughout the cycle with a dramatic rise occurring 2-4 days after ovulation; this rise may be caused by the

preovulatory surge---in oestradiol concentrations (Bonney, 1985). PLA_2 activity in the human endometrium then decreases during the secretory phase, when progesterone concentrations are high, and is low at menstruation. Therefore, PLA_2 activity in the uterus appears to be stimulated by oestradiol when progesterone concentrations are low, but oestradiol cannot stimulate PLA_2 activity when progesterone levels are high. Previous studies in bovine seminal vesicles have shown that the formation of specific prostaglandins is influenced by substrate concentration (Flower, Cheung and Cushman, 1973). PGE_2 formation was found to be maximal at a relatively low concentration of arachidonic acid, and was inhibited by higher concentrations of arachidonic acid which produced maximal stimulation of $PGF_{2\alpha}$. Thus, increasing concentrations of oestradiol prior to luteolysis in the guinea-pig could stimulate endometrial PLA_2 resulting in an increase in intracellular levels of free arachidonic acid and the specific stimulation of $PGF_{2\alpha}$ formation. Removal of endogenous steroids by ovariectomy resulted in a 10-fold increase in PGE_2 levels in the rat uterus and this stimulation was abolished by oestradiol treatment (Ham, Cirillo, Zanetti and Kuehl, 1975). However, oestradiol treatment stimulated PGF levels in the ovariectomised rat uterus, an effect which was blunted by the co-administration of progesterone with oestradiol. Addition of excess arachidonic acid to human endometrial and decidual cells resulted in a disproportionate increase in PGE_2 synthesis compared to $PGF_{2\alpha}$ synthesis (Smith and Kelly, 1987), and exogenous arachidonic acid caused a much greater stimulation of PGE_2 from the uterus of the guinea-pig than was caused by endogenous arachidonic acid released by the action of A23187 (Poyser, 1985a). These results suggest that the synthesis of

specific prostaglandins is mediated differently by exogenous and endogenous arachidonic acid. As endogenous arachidonic acid released by A23187 produces a much greater stimulation of synthesis of $PGF_{2\alpha}$ than of PGE_2 (Poyser and Brydon, 1983), the hypothesis that stimulation of endometrial PLA_2 would result in a specific increase in $PGF_{2\alpha}$ formation is feasible.

The studies on steroidal control of PLA_2 activity in the uterus suggested that continued secretion of progesterone by the corpus luteum during pregnancy could prevent the increase in endometrial $PGF_{2\alpha}$ release at the end of the cycle by inhibiting the stimulation of endometrial PLA_2 by oestradiol and thus prevent the rise in intracellular free arachidonic acid levels. This may be due to progesterone inhibiting the synthesis of the oestradiol-stimulated protein which is responsible for the increase in $PGF_{2\alpha}$ output at the end of the cycle. However, in the guinea-pig a significant difference in the levels of plasma progesterone between the pregnant and non-pregnant animal is not seen until after Day 12, whereas levels of $PGF_{2\alpha}$ in the utero-ovarian vein rise on Day-10 in non-pregnant but not in pregnant animals (Antonini et al., 1976). Therefore in the pregnant guinea-pig, an increase in luteal progesterone synthesis does not precede the failure of endometrial $PGF_{2\alpha}$ levels to rise and a luteotrophic factor from the conceptus is unlikely to be the direct cause of prevention of luteal regression in the pregnant guinea-pig.

Experiments in this thesis have shown that the guinea-pig conceptus does not secrete any antiviral activity indicative of interferon-like proteins, and that human α-interferon does not inhibit prostaglandin output from Day-15 non-pregnant guinea-pig endometrium in culture for 24h. Consequently, it appears that the

antiluteolytic factor produced by the guinea-pig conceptus is not an interferon and therefore is not similar to either oTP-1 or bTP-1, which are the antiluteolytic proteins secreted by the sheep and cow conceptus, respectively. There are several differences between pregnant guinea-pigs and pregnant sheep which indicate that the guinea-pig antiluteolytic factor is likely to be different from oTP-1. Firstly, implantation takes place in guinea-pigs 7 days after conception which is before endometrial $PGF_{2\alpha}$ synthesis has to be prevented, whereas in sheep implantation takes place several weeks after conception which is after inhibition of endometrial $PGF_{2\alpha}$ synthesis has occurred. Secondly, in unilaterally pregnant guinea-pigs with the non-pregnant horn isolated at the cervical end (i.e. there is no connection between the two horns), endometrial $PGF_{2\alpha}$ synthesis is reduced in the non-pregnant horn and the corpora lutea are maintained (Poyser and Maule Walker, 1979). Also, transplantation of the conceptuses to the spleen prevents luteal regression (Bland and Donovan, 1969b). Both these studies indicate that the antiluteolytic factor from guinea-pig conceptuses acts systemically. However, in bilaterally ovulated, unilaterally pregnant sheep with the non-pregnant horn isolated from the pregnant horn, corpora lutea in the ovary adjacent to the non-pregnant horn regress (Moor, 1968) indicating that $PGF_{2\alpha}$ synthesis in the non-pregnant horn has not been inhibited. This suggests that oTP-1 does not circulate nor acts systemically and, in fact, oTP-1 is not detectable in the uterine venous blood of pregnant sheep nor is it found in the isolated non-pregnant horn of unilaterally pregnant sheep (Kazemi et al., 1988).

The human is more like the guinea-pig than the sheep since implantation in women occurs 7 days after conception, and the

substance which inhibits endometrial prostaglandin synthesis acts systemically since endometrial prostaglandin levels are low in women with ectopic pregnancy (Abel, Smith and Baird, 1980). In addition α-interferon is unable to inhibit the output of $PGF_{2\alpha}$ from human endometrial tissue (Mitchell and Smith, 1989).

The type of implantation which occurs in various species may also affect the mechanism of maternal recognition of pregnancy. Pigs, horses and ruminants exhibit epithelio-chorial implantation in which all the tissues of the uterus remain intact and the foetal blood never mixes with the maternal blood. However, both rodents, including the guinea-pig, and primates, including the human, exhibit a haemo-chorial type of placentation in which the epithelium is destroyed and the maternal blood passes through lacunae formed in the trophoblast. This is the closest form of placentation which occurs and involves direct mixing of maternal and conceptual blood. As the guinea-pig conceptus implants at Day 7 and differences in the levels of $PGF_{2\alpha}$ in the utero-ovarian vein between pregnant and non-pregnant guinea-pigs are evident by Day 10 (Antonini et al.,1976), it may be that the guinea-pig conceptus secretes an antiluteolytic substance directly into the maternal vasculature. This would fit with the systemic action of the antiluteolytic factor in the guinea-pig. The domestic ruminants such as the cow, sheep and goat have not implanted prior to maternal recognition of pregnancy and therefore the only way to signal their presence to the mother is by the secretion of some diffusable substance into the uterine lumen from where it may reach the endometrium and inhibit $PGF_{2\alpha}$ release. Experiments carried out in this thesis have shown that substances secreted by the Day-15 guinea-pig conceptus are unlikely to mediate the antiluteolytic effects of the guinea-pig conceptus as the output

of prostaglandins from Day-15 guinea-pig endometrium in culture is unaffected by either co-culture with guinea-pig conceptus tissue or the medium in which Day-15 conceptuses have been incubated. Neither did proteins obtained from the medium of Day-15 conceptuses at a concentration of 500μg/ml affect endometrial prostaglandin output in the Day-15 guinea-pig. A low molecular weight protein fraction purified from the medium of Day-15 conceptuses did inhibit prostaglandin output from Day-15 endometrium after 6h of culture but the effect was small and was not continued to 12h of culture. As this protein was present at a concentration of 25μg/ml and oTP-1 inhibits $PGF_{2\alpha}$ and PGE release from cultured ovine endometrial cells at a concentration of only 30ng/ml (Salamonsen et al., 1988), it appears unlikely that this protein is involved in mediating the sustained suppression of $PGF_{2\alpha}$ synthesis in the pregnant guinea-pig. Neither do proteins purified from the Day-15 guinea-pig conceptus affect the activity of cobra venom PLA_2 in an in vitro assay. Therefore by Day-15 of pregnancy, the guinea-pig conceptus does not produce secretable substances during culture which affect endometrial prostaglandin production. It would be interesting to investigate whether earlier guinea-pig conceptuses secrete factors which are capable of inhibiting endometrial prostaglandin synthesis. Indeed, $PGF_{2\alpha}$ output by the guinea-pig uterus superfused in vitro has been shown to be already significantly lower on Day 7 of pregnancy than on Day 7 of the cycle (Poyser, 1984a). In addition, $PGF_{2\alpha}$ production by homogenates of guinea-pig endometrium was lower on Day 7 of pregnancy than on Day 7 of the cycle (Maule Walker and Poyser, 1973, 1974). Therefore the guinea-pig conceptus influences uterine prostaglandin production as early as Day 7 of pregnancy and consequently maternal recognition of pregnancy in the

guinea-pig may even occur during the preimplantation period.

Preimplantation signals between the embryo and the mother have been shown to exist in mice. Between mating and implantation, a significant thrombocytopenia (a reduction in the number of platelets) occurs in mice with fertilized ova in the reproductive tract (O'Neill, 1985a). Therefore, a physiological response can be induced in the mother even before the mouse conceptus has implanted. Injection of embryo culture medium into splenectomized mice induced thrombocytopenia and the dose response curve to the embryo culture medium was similar to that seen with platelet-activating factor (PAF)(O'Neill, 1985b). PAF is a potent lipid mediator which has been chemically identified as 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine. The platelet-activating factor from the mouse embryo was also found to have similar biochemical and physiological characteristics to PAF (O'Neill, 1985c). An increased vascular demand for blood platelets, resulting in mild thrombocytopenia, was also seen as the initial response to pregnancy in women (O'Neill, Pike, Porter, Gidley-Baird, Sinosich and Saunders, 1985). In addition, significant levels of PAF have been found in the preimplantation pregnant rabbit endometrium (Angle, Jones, Pinckard, McManus and Harper, 1985) and the normal rat uterus (Yasuda, Satouchi and Saito, 1986). PAF was therefore proposed to be an early embryonic message which might be involved in the prevention of luteal regression. Indeed, O'Neill (1987) showed that an enhanced progesterone production occurred in human granulosa cells <u>in vitro</u> in the presence of PAF. However the PAF antagonist SRI 63-441 had no effect on peripheral blood progesterone concentrations between Day 1 and Day 9 of pregnancy in mice (Spinks and O'Neill, 1988).

Peripheral progesterone concentrations are not different in the

pregnant guinea-pig on Day 7 compared to the non-pregnant guinea-pig on Day 7 (Poyser, 1984a), and consequently any preimplantation factor produced by the guinea-pig conceptus cannot be acting by a luteotrophic effect on the corpus luteum but would have to prevent luteal regression by a direct effect on uterine $PGF_{2\alpha}$ synthesis. However, PAF was found to stimulate prostaglandin output from the superfused guinea-pig uterus (Norman and Poyser, unpublished observations) and is therefore an unlikely candidate for a preimplantation antiluteolytic factor from the guinea-pig conceptus. In contrast to the effect of PAF in the guinea-pig, PAF stimulates PGE_2 in human glandular endometrium without affecting levels of $PGF_{2\alpha}$ (Smith and Kelly, 1988) while, in the presence of oestradiol, PAF increases PGE_2 and decreases $PGF_{2\alpha}$ in human glandular endometrium (Alecozay, Harper, Schenken and Hanahan, 1991). In addition, PAF inhibits oxytocin-induced $PGF_{2\alpha}$ release in the sheep uterus (Battye, O'Neill and Evans, 1990). Therefore, these results suggest that PAF may play a role in the prevention of luteal regression during pregnancy in some species. However, the stimulation of prostaglandin release from the Day-7 guinea-pig uterus by PAF was not large and may have been due to PAF-induced formation of IP_3 and release of calcium which has been shown to occur in human secretory endometrium (Ahmed and Smith, 1991) with the resultant activation of PLA_2.

In the cow, jugular vein plasma progesterone concentrations are higher in the pregnant than in the non-pregnant animal as early as Day 10 (Lukaszewska and Hansel, 1980). Culture medium harvested from Day 13-18 cow conceptus tissue was able to stimulate progesterone synthesis in dispersed bovine luteal cells (Hickey and Hansel, 1987). The luteotrophic factor from the cow conceptus was found to

be a small (Mr < 10,000 Da), heat-labile, lipid-soluble substance. However, this factor is not absolutely essential for corpus luteum maintenance in the pregnant cow as the transfer of Day-16 conceptuses to non-pregnant recipients results in the maintenance of the corpus luteum (Betteridge et al., 1980) even though the recipient has not been exposed to the luteotrophic embryonic secretion prior to this time. Platelet counts were found to be low in pregnant cows compared to non-pregnant cows between Days 7 and 16 (Hansel, Stock and Battista, 1989), indicating that thrombocytopenia is an early maternal response to pregnancy in the cow as well as the mouse and human. However, PAF did not affect either basal or LH-stimulated progesterone synthesis in dispersed bovine luteal cells (Hansel et al., 1989) and is therefore not responsible for the luteotrophic activity of cow conceptus culture medium. Co-incubation of platelets with dispersed bovine luteal cells did stimulate progesterone synthesis and this stimulation was independent of whether the platelets had been preactivated with PAF. Subsequent experiments using the 5-hydroxytryptamine (serotonin) receptor antagonist mianserin, indicated that serotonin is the major platelet-derived compound responsible for platelet-induced stimulation of luteal progesterone synthesis. Therefore, PAF and platelet-derived factors may be the luteotrophic or antiluteolytic factors produced by the conceptus of a number of animal species.

Proteins known to have an inhibitory effect on PLA_2 activity include the glucocorticoid hormones which are secreted by the adrenal cortex. In 1979, Flower and Blackwell proposed that anti-inflammatory steroids (i.e. glucocorticoids) blocked prostaglandin synthesis in the guinea-pig lung by causing cells to synthesise and release a polypeptide with anti-phospholipase

properties. Several glucocorticoid induced proteins were found which exerted an anti-inflammatory effect by inhibiting phospholipase activity including lipomodulin, a 40kDa protein isolated from rabbit peritoneal neutrophils (Hirata, Schiffman, Venkatsubramanian, Solomon and Axelrod, 1980), macrocortin, a 15kDa protein isolated from rat peritoneal macrophages (Blackwell, Carnuccio, Di Rosa, Flower, Parente and Persico, 1980), and renocortin, a 15kDa protein isolated from rat renomedullary interstitial cells (Cloix, Colard, Rothhut and Russo-Marie, 1983). Comparison of the properties of lipomodulin, macrocortin and renocortin led to the conclusion that these proteins were functionally identical entities and that the observed discrepancy in molecular weight was caused by proteolysis. It was proposed that the family of proteins be known as lipocortins, which reflects their activity as modulators of lipid metabolism and their intimate relationship with hormones of the adrenal cortex (Di Rosa, Flower, Hirata, Parente and Russo-Marie, 1984).

Lipocortins also share consensus sequences with a family of calcium-binding proteins including calelectrin (34kDa) from the ray Torpedo marmorata, endonexin (32.5kDa) from adrenal medulla and liver, and protein II (32kDa) from the brush border of intestinal epithelium (Kretsinger and Creutz, 1986). In addition p36/protein I/ calpactin I (36kDa), the substrate of several tyrosine protein kinases, has homologous sequences to lipocortin and inhibits bovine pancreas PLA_2 with a similar potency to lipocortin (Khanna, Hee-Chong, Severson, Tokuda, Chong and Waisman, 1986). Endonexin/ protein II (32.5kDa) from bovine liver also inhibits the activity of PLA_2 from the porcine pancreas (Fauvel, Salles, Roques, Chap, Rochat and Douste-Bizay, 1987). Thus, it appears that lipocortins and calcium-binding proteins (calpactins) are both structurally and

functionally homologous. In vitro inhibition of PLA_2 by lipocortins/calpactins is due to sequestering of the phospholipid substrate by the inhibitor protein, rather than a direct interaction with PLA_2 (Davidson, Dennis, Powell and Glenney, 1987). Lipocortin has been shown to be secreted by human endometrium in culture, and the production of lipocortin in both proliferative and secretory endometrium is stimulated by treatment with the glucocorticoid, dexamethasone (Gurpide, Markiewicz, Schatz and Hirata, 1986). Consequently, lipocortins may control prostaglandin synthesis in the uterus. However, dexamethasone only inhibited $PGF_{2\alpha}$ output from secretory endometrium and not from proliferative endometrium, even though dexamethasone increases the output of lipocortin from both tissues (Schatz, Markiewicz and Gurpide, 1986; Kelly and Smith, 1987). The main glucocorticoid hormone secreted by the guinea-pig is hydrocortisone (Fajer and Vogt, 1963). However, hydrocortisone had no effect on either the basal or A23187-stimulated output of prostaglandins from Day-15 guinea-pig uterus superfused in vitro (Poyser, 1987b), nor on the basal output of prostaglandins from Day-15 guinea-pig endometrium in culture (Riley and Poyser, 1987b). Therefore, it is unlikely that lipocortin-like proteins are involved in the control of prostaglandin synthesis in the guinea-pig uterus.

Uteroglobin or blastokinin is a small (16kDa) progesterone-induced protein which was originally identified as being secreted by the rabbit uterus during early pregnancy (Beier, 1968; Krishnan and Daniel, 1967). Uteroglobin is also similar in structure to lipocortin and inhibits the activity of PLA_2 (Levin, Butler, Schumacher, Wightman and Mukherjee, 1986; Miele, Cordella-Miele, Facchiano and Mukherjee, 1988). A protein which is immunologically similar to uteroglobin has been found in human endometrial tissue

from the mid- and late-luteal phase (Kikuwa, Cowan, Tejada and Mukherjee, 1988), and consequently may be involved in regulating endometrial $PGF_{2\alpha}$ synthesis in the human. Two inhibitors (I and II) of PLA_2 have also been found in amniotic fluid from non-labouring women at term (Wilson, Liggins, Aimer and Skinner, 1985). These compounds inhibited arachidonic acid release and PGF synthesis in dispersed endometrial cells stimulated by histamine or bradykinin. Inhibitor II was found to be a 58-60kDa protein with a pI of 8.4 (Wilson, Liggins and Joe, 1989). The protein named gravidin inhibited arachidonic acid release in response to histamine or calcium ionophore in a dispersed decidual cell system and in a cell-free assay of porcine PLA_2. Gravidin has subsequently been shown to be identical to secretory component of IgA (Wilson and Christie, 1991). A PLA_2 inhibitor with a similar molecular weight to gravidin was found in porcine serum (Nevalainen and Evilampi, 1984). Therefore, PLA_2 inhibitory proteins exist in a variety of tissues.

It is concluded from experiments in this thesis that prevention of luteal regression in the pregnant guinea-pig is not due to the production of interferon-like, antiluteolytic proteins by the conceptus, as has been found in the sheep, cow and goat. It appears likely that maternal recognition of pregnancy in the guinea-pig occurs earlier in gestation, perhaps even in the pre- or peri-implantation period (Day 7), than in domestic ruminants as factors produced by the Day-15 conceptus in culture have no effect on either endometrial prostaglandin synthesis or PLA_2 activity. Further investigations are required to determine the character and nature of the antiluteolytic factor produced by the guinea-pig conceptus.

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